Universidad Autónoma de Madrid

Facultad de Ciencias

Departamento de Biología Molecular

ROLE OF THE NuA4 COMPLEX IN THE REGULATION OF FLOWERING TIME IN *Arabidopsis thaliana*

Doctoral Thesis

Alfonso Mouriz Villar

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ROLE OF THE NuA4 COMPLEX IN THE REGULATION OF FLOWERING TIME IN *Arabidopsis thaliana*

Alfonso Mouriz Villar

Licenciado en Bioquímica

Presenta esta memoria para optar al grado de Doctor en Bioquímica, Biología Molecular, Biomedicina y Biotecnología.

Este trabajo ha sido realizado en el Centro de Biotecnología y Genómica de Plantas (UPM-INIA) bajo la dirección y supervisión de los doctores José Antonio Jarillo Quiroga y Manuel Ángel Piñeiro Galvín.

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José Antonio Jarillo Quiroga, Investigador Titular de OPIS en el Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)

CERTIFICA

Que la Tesis Doctoral titulada “Role of the NuA4 complex in the regulation of flowering time in Arabidopsis” ha sido realizada bajo su supervisión en el Centro de Biotecnología y Genómica de Plantas (UPM-INIA).

El trabajo realizado por Alfonso Mouriz Villar reúne todas las condiciones requeridas por la legislación vigente, así como la originalidad y calidad científica para poder ser presentado y defendido con el fin de optar al grado de Doctor.

Y para que conste donde proceda, firmo el presente certificado.

Madrid a 5 de noviembre de 2015

Dr. José Antonio Jarillo Quiroga
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Co-director de la Tesis

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SUMMARY

As sessile organisms, plants have to face the challenges of a continuously changing environment, and as a result, they have developed complex adaptation mechanisms that allow them to respond to these challenges and maximize their fitness. One example is the time of flowering, which must be precisely regulated in order to ensure the reproductive success of plant species. Chromatin remodeling plays a crucial role in the establishment and maintenance of gene expression patterns that drive developmental transitions in plants, and it is of utmost importance in the control of master regulators of the floral transition, including the floral repressor *FLOWERING LOCUS C* (*FLC*). In particular, the exchange of histone H2A by the histone variant H2A.Z in the *FLC* chromatin mediated by the SWR1 complex is required for the transcriptional activation of this floral repressor. In yeast, the SWR1 complex shares four subunits with the histone acetyltransferase complex NuA4, and a close functional relationship between these complexes in the control of gene expression has been described. Most of the 13 subunits of the *Saccharomyces cerevisiae* NuA4 complex are conserved in Arabidopsis, suggesting that an interaction between these two complexes could exist in Arabidopsis, and that histone acetylation mediated by this putative NuA4 complex may also have a role in the regulation of flowering time.

The Arabidopsis homologues of the yeast Esa1 (AtHAM1 and AtHAM2) and Yaf9 (AtYAF9a) subunits regulate H4 acetylation levels at the *FLC* locus. In this work, we have characterized the roles of some of the Arabidopsis NuA4 subunits, paying special attention in their roles in flowering time. We have revealed a role for Tra1, Epl1 and Eaf6 homologues as activators of the floral transition through the regulation of the flowering master genes *FLC*, *FT* and *SOC1*. We have also shown that the Arabidopsis homologues of Eaf3, MRG1 and MRG2, play redundant roles in the activation of flowering under LD by inducing the expression of *FT*. MRG1 and 2 are also involved in the activation of *FLC*. The direct binding of MRG2 to the chromatin of *FLC* suggests that MRG1 and MRG2 are involved in the chromatin-mediated regulation of this master regulator of flowering. Also, global transcriptomic studies suggest that MRG1 and MRG2 are also involved in the regulation of many biological processes, such as defense against pathogens.

We have also shown that ING1 and ING2, the Arabidopsis homologues of Yng2, play opposite roles in the regulation of flowering. Both proteins bind the chromatin of *FLC* and are required to maintain proper H4 acetylation levels in this locus. ING2 also binds and regulates H4 acetylation in the chromatin of *FT*. Their ability to physically interact with NuA4 components suggests that ING1 and ING2 regulate the acetylation status of their target genes in the context of this multiprotein complex. Transcriptomic data regarding *ing1* and *ing2* mutants indicate that ING1 and ING2 have independent and redundant functions in the control of gene expression in Arabidopsis, and suggest a role for them as central regulators of several biological processes.

Altogether, our results support a role for the putative Arabidopsis NuA4 complex in the regulation of the floral transition.

RESUMEN

Las plantas han desarrollado complejos mecanismos de adaptación que les permiten responder y adaptarse a las condiciones cambiantes del medio que las rodea, modulando su desarrollo para maximizar su supervivencia. Por ejemplo, el tiempo de floración está muy finamente regulado para garantizar el éxito reproductivo de especies vegetales. Los mecanismos de remodelación de la cromatina juegan un papel fundamental en el establecimiento y mantenimiento de los patrones de expresión que controlan las transiciones de fase en el desarrollo vegetal, particularmente en el control de la expresión de genes maestros de la transición floral, como el represor floral *FLOWERING LOCUS C* (*FLC*). El intercambio de la histona H2A por la variante histónica H2A.Z en la cromatina de *FLC* mediado por el complejo SWR1 es necesario para la activación transcripcional de este gen. En levaduras, el complejo SWR1 comparte cuatro subunidades con el complejo histona acetil-transferasa NuA4, y una estrecha relación funcional entre ambos complejos se ha descrito en el control de la expresión génica. La mayoría de las 13 subunidades que componen el complejo NuA4 de *Saccharomyces cerevisiae* están conservadas en Arabidopsis, lo que sugiere que podría existir una interacción entre estos complejos y que la acetilación mediada por un posible complejo NuA4 podría tener un papel en la regulación del tiempo de floración en Arabidopsis.

Los homólogos de las subunidades Esa1 y Yaf9 en Arabidopsis, AtHAM1/2 y AtYAF9a regulan los niveles de acetilación de histonas en *FLC*, y la pérdida de función de dichos homólogos causa una alteración del tiempo de floración. En esta Tesis Doctoral hemos caracterizado a nivel molecular y funcional algunas subunidades del complejo NuA4 de Arabidopsis, con especial atención a su participación en la regulación del tiempo de floración. Hemos desvelado un papel como activadores de la transición floral para los homólogos de Tra1, Epl1 y Eaf6 en Arabidopsis a través de la regulación de *FLC*, *FT* y *SOC1*, y hemos demostrado que los homólogos de Eaf3, MRG1 y MRG2, tienen funciones redundantes en la activación floral en día largo, induciendo la expresión de *FT*. MRG1 y MRG2 también están implicadas en la activación de *FLC*. La unión directa de MRG2 a la cromatina de este gen sugiere que las proteínas MRG participan en la regulación epigenética de este represor floral. Además, nuestros análisis transcriptómicos sugieren que las proteínas MRG están implicadas en la regulación de múltiples procesos biológicos, tales como la defensa frente a patógenos.

Por otro lado, hemos demostrado que ING1 e ING2, los homólogos de Yng2, tienen funciones opuestas en el control del tiempo de floración en Arabidopsis. Ambas proteínas se unen a la cromatina de *FLC* y son necesarias para mantener niveles adecuados de acetilación de H4 en este locus. Además, ING2 también se une y regula los niveles de acetilación de H4 en la cromatina de *FT*. Tanto ING1 como ING2 interaccionan con otros componentes de NuA4, lo que sugiere que estas proteínas regulan el nivel de acetilación de sus genes diana en el contexto de este complejo. Nuestros datos transcriptómicos indican que ING1 e ING2 tienen funciones independientes y redundantes en el control de la expresión génica, y son importantes reguladores de diversos procesos biológicos.

En conjunto, estos resultados apoyan el papel de un posible complejo NuA4 en la regulación de la transición floral en Arabidopsis.

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ABBREVIATIONS

ABRC: Arabidopsis Biological Resource Center of Ohio State

ATP: adenosine triphosphate

bp: base pairs

BSA: bovine serum albumin

cDNA: complementary deoxyribonucleic acid

CDS: coding sequence

DNA: deoxyribonucleic acid

DNAse: deoxyribonuclease

EDTA: ethylenediaminetetraacetic acid

GA: gibberellic acid

GFP: Green fluorescent protein

GM: germination medium

h: hours

INDEL: insertion/deletion polymorphism

Kb: Kilobase

KDa: Kilodalton

LB: Lysogeny broth

LD: long day

M: mitosis

min: minute

MOPS: 3-(*N*-morpholino)propanesulfonic acid

mRNA: messenger ribonucleic acid

MS: Murashige and Skoog

n.a.: not analyzed

NASC: Nottingham Arabidopsis Stock Center

PAGE: Polyacrylamide gel electrophoresis

PCR: polymerase chain reaction

qPCR: quantitative real time polymerase chain reaction.

rpm: revolutions per minute

s: seconds

SD: short day

SDS: sodium dodecyl sulfate

TBE: Tris/Borate/EDTA

T-DNA: transferred DNA

Tris: Tris(hydroxymethyl)aminomethane

UTR: untranslated region

v/v: volume/volume

wt: wild type

Y2H: Yeast two- hybrid

ZT: Zeitgeber time

µE m^{-2} s^{-1}: µEinsteins m^{-2} s^{-1}

INTRODUCTION

INTRODUCTION

1. The floral transition

Plant development is determined by the activity of the meristems, groups of pluripotent cells that divide and differentiate into plant organs (Shen and Xu, 2009). The shoot apical meristem (SAM) is responsible for the formation of the aerial part of the plant, while the root apical meristem (RAM) gives rise to the root system.

Plants are sessile organisms that have the ability to perceive multiple environmental cues and adjust their development to the changing environment that surrounds them (Srikanth and Schmid, 2011). The floral transition is the switch from vegetative to reproductive development and is a very finely regulated process, as it determines the reproductive success of plants (Amasino, 2010). This developmental transition involves important changes in the SAM identity. During the vegetative phase, the SAM produces leaves and axillary meristems, which in turn will produce vegetative branches. After the floral transition, flowers start to develop from the reproductive meristems (Coen and Meyerowitz, 1991).

Flowering time is controlled by a high number of factors, both endogenous and exogenous. Endogenous factors mainly depend on the developmental stage of the plant, while environmental factors include the photoperiod (the ratio between daily periods of light and darkness), light quality and intensity, and temperature (Kim et al., 2009; Michaels, 2009; Amasino, 2010; Imaizumi, 2010). Specifically, the acceleration of flowering resulting from the prolonged exposure to low winter temperatures is called vernalization and has a great influence on the life history of plant species and varieties (Schmitz and Amasino, 2007; Kim et al., 2009).

Based on their photoperiodic response, plants can be divided into three different groups (Jarillo et al, 2008; Andres and Coupland, 2012):

- Long day (LD) plants, in which flowering is induced when the light period is longer than the dark period. This is the case of oat (*Avena sativa*).
- Short day (SD) plants, in which flowering is promoted when the light period is shorter than the dark period, such as maize (*Zea mays*) and rice (*Oryza sativa*)
- Plants insensitive to photoperiod, such as tomato (*Solanum lycopersicum*).

Likewise, some plants present an absolute vernalization requirement for flowering, such as beet plants (*Beta vulgaris*), while others respond to vernalization without an obligate requirement or do not respond at all, like several cereal species (Kim et al., 2009).

2. Genetic control of flowering in *Arabidopsis thaliana*

Most of our current understanding on how the floral transition is regulated comes from studies in the model species *Arabidopsis thaliana*. During the vegetative phase,

Arabidopsis thaliana grows as a rosette, forming leaves without elongating the internodes. After the induction of the floral transition, the internodes elongate, the leaves of the main stem (cauline leaves) develop and the reproductive meristems give rise to flowers and form an inflorescence (Coen and Meyerowitz, 1991; Kim et al., 2009).

Arabidopsis is an annual facultative LD plant; it does not show an absolute photoperiod requirements to flower, but reproductive growth is accelerated in LD conditions. Moreover, some Arabidopsis ecotypes can respond to vernalization (He, 2012).

2.1. Activation of flowering

The analysis of natural variation and the characterization of Arabidopsis mutants affected in flowering time over recent years has led to the identification of different genetic regulators of the floral transition, and to the establishment of a series of floral promoting pathways that are intertwined and form a complex regulatory network that precisely modulates the time when the floral transition takes place (Baurle and Dean, 2006; Turck et al., 2008; Amasino and Michaels, 2010; Andres and Coupland, 2012; He, 2012).

The time of flowering is determined by the activity of several pathways that respond to both environmental and internal cues (Jarillo and Pineiro, 2011). These pathways converge in the regulation of a few master genes, the so-called floral integrators. The main floral integrators are *FLOWERING LOCUS T* (*FT*), *TWIN SISTER OF FT* (*TSF*), and *SUPPRESSOR OF OVEREXPRESSION OF CO1/AGAMOUS-LIKE 20* (*SOC1/AGL20*), *AGAMOUS-LIKE 24* (*AGL24*) and *LEAFY* (*LFY*) (Fornara et al., 2010). These floral integrators activate the expression of the floral meristem identity genes *APETALA 1* (*AP1*), *AP2*, *CAULIFLOWER* (*CAL*), *FRUITFULL* (*FUL*) and *LFY*, which in turn activate the expression of the floral organ identity genes, such as *AP1*, *AP3*, *PISTILLATA* (*PI*), *AGAMOUS* (*AG*) and *SEPALLATA 1-4* (*SEP1-4*) (Wigge et al., 2005; Jaeger et al., 2006; Turck et al., 2008; Causier et al., 2010).

2.1.1. Gene regulatory networks that control flowering induction

2.1.1.1. Photoperiod

Plants need to measure changes in day length in order to adapt their flowering responses to the photoperiod. To this end, plants integrate the light information they perceive through photoreceptors with an internal rhythm provided by the circadian clock, and this mediates several responses, including flowering time (Imaizumi, 2010). This internal rhythm, consisting of a number of positive and negative feedback loops between proteins expressed in the morning and in the evening, regulates the expression of many genes with an oscillation period of nearly 24 hours (de Montaigu et al., 2010; Imaizumi, 2010; Jarillo and Pineiro, 2011). This mechanism eventually regulates the expression pattern of *CONSTANS* (*CO*), the main component of the flowering promoting photoperiod pathway (Fig. 1). *CO* is responsible for the activation of *FT* and its homolog *TSF* in the vasculature of the leaves (Jarillo and Pineiro, 2011). The FT protein moves through the phloem to the SAM to activate the expression of meristem identity genes and promote flowering (Putterill et al., 2004; Baurle and Dean, 2006; Jarillo and Pineiro, 2011) (Fig. 1). In LD, the peak of expression of *CO* overlaps with the light period, which

allows stabilization of the CO protein. This leads to the activation of *FT* by CO and the induction of flowering. Conversely, under SD, *CO* is only expressed in the dark period and, in these conditions, the CO protein is degraded, keeping a low expression of *FT* and delaying flowering (de Montaigu et al., 2010).

The expression of *CO* is transcriptionally regulated by multiple factors such as *GIGANTEA* (*GI*), *FLAVIN BINDING, KELCH REPEAT AND F-BOX1* (*FKF1*), and members of the *CYCLING DOF FACTOR* family (*CDFs*), among others (Andres and Coupland, 2012). The CDFs are transcriptional repressors of *CO*. Under LD, the E3 ubiquitin ligase FKF1 interacts with GI and this light-dependent interaction triggers the degradation of the CDFs and releases the repression of *CO* (Sawa et al., 2007). In SDs, the interaction between GI and FKF1 is not stable and leads to the accumulation of the CDFs and the transcriptional repression of *CO* (Sawa et al., 2007).

Apart from its transcriptional regulation, the stability of the CO protein is also controlled by protein complexes that modulate its proteasome-mediated degradation. The complex formed by the E3 ubiquitin ligase CONSTITUTIVE MORPHOGENIC 1 (COP1) and the SUPPRESSOR OF PHYTOCHROME A-105 (SPA) proteins degrades CO in darkness (Chen et al., 2010; Jarillo and Pineiro, 2011), while the E3 ubiquitin ligase HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 1 (HOS1) participates in the morning degradation of CO (Lazaro et al., 2012). Thus, dark conditions promote the degradation of CO and delay flowering. Only when the peak of expression of *CO* overlaps with the light period (in LD conditions), the CO protein is stable and can activate *FT*, allowing the acceleration of flowering under long photoperiods (Andres and Coupland, 2012). In this way, LD-dependent activation of the floral integrator gene *FT* is ensured under optimal environmental conditions for flowering (Lazaro et al., 2012).

2.1.1.2. Vernalization

Some Arabidopsis accessions display a flowering response to the exposure to prolonged periods of cold of winter, a process known as vernalization (He, 2012). The vernalization requirement prevents some Arabidopsis accessions from flowering in autumn, when the environmental conditions are unfavorable, and facilitates the floral transition in the spring. In Arabidopsis, the vernalization requirement depends on the dominant alleles of *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*) and therefore, those accessions with functional alleles of both genes show a vernalization response (Crevillen and Dean, 2011). *FRI* encodes a plant-specific protein that strongly up-regulates *FLC* (Crevillen and Dean, 2011), while *FLC* encodes a MADS domain transcription factor that acts as a repressor of flowering (Sheldon et al., 2000; Michaels and Amasino, 2001) by negatively regulating the floral integrators *FT* and *SOC1* (Helliwell et al., 2006; Searle et al., 2006). The rapid-cycling accession Columbia (Col), normally used in the laboratory, carries an active allele of *FLC* and a non-functional *FRI* (Gazzani et al., 2003; Shindo et al., 2005), whereas in Landsberg *erecta* (L*er*), neither *FLC* nor *FRI* are functional (Gazzani et al., 2003; Michaels et al., 2003).

Even though *FLC* is the main responsible gene for the vernalization response in Arabidopsis, *flc-3* null mutant plants can still respond to vernalization (Michaels and Amasino,

2001). There are five MADS box paralogs of *FLC* in Arabidopsis, called *MADS AFFECTING FLOWERING1*/*FLOWERING LOCUS M* (*MAF1*/*FLM*), *MAF2*, *MAF3*, *MAF4* and *MAF5* (Ratcliffe et al., 2001; Ratcliffe et al., 2003). At least *MAF1*/*FLM* and *MAF2* act as floral repressors, and all of them are regulated by vernalization, which might explain why *flc* null plants still show a vernalization response (Ratcliffe et al., 2003; Sheldon et al., 2009; Zografos and Sung, 2012).

Vernalization stably represses *FLC* upon cold exposure through the introduction of a number of epigenetic modifications in the chromatin of this gene. This process is quantitative, since the duration of the cold exposure correlates with the degree of silencing of *FLC*. It is also mitotically stable, and it is maintained after plants return to warm conditions (Song et al., 2013).

The isolation of mutants with an impaired vernalization response has led to the identification of genes involved in this process (Kim et al., 2009). Among them, we can find members of the *VERNALIZATION5*/*VIN3-LIKE* (*VEL*) family, which encode PHD-containing proteins. This family includes *VERNALIZATION INSENSITIVE 3* (*VIN3*) and its homologs *VIN3-LIKE1*/*VERNALIZATION5* (*VIL1*/*VRN5*), *VIL2*/*VEL1* and *VIL3*/*VEL2*. *VERNALIZATION2* (*VRN2*) encodes a member of the Polycomb group (PcG) of proteins and it is also involved in the vernalization process. Given the importance of the epigenetic regulation of vernalization, this process will be discussed later.

2.1.1.3. Ambient temperature

Growing temperatures have a huge impact on flowering (Fig. 1). In fact, Arabidopsis plants can detect growing temperature differences as small as 1ºC (Kumar and Wigge, 2010). Arabidopsis flowers earlier at warm growing temperatures of 27-28ºC, compared with standard growing temperatures of 22ºC. Recent studies have contributed to elucidate the molecular basis of this phenomenon (Wigge, 2013; Verhage et al., 2014; Capovilla et al., 2015). The chromatin remodeling complex SWR1, which catalyzes the exchange of histone H2A by the histone variant H2A.Z seems to play a role in the thermosensory regulation of flowering (Kumar and Wigge, 2010; Jarillo and Pineiro, 2015). *actin-related protein 6 (arp6)* mutant plants, deficient in a SWR1 component, phenocopy plants grown at warm temperatures, and that led to propose a role for H2A.Z in this process (Kumar and Wigge, 2010). At cooler temperatures of 22ºC, this histone variant is present around the transcription start site in the chromatin of *FT*, negatively regulating its expression. Warm temperatures promote the eviction of nucleosomes containing H2A.Z in the chromatin of *FT*, allowing its transcriptional activation and promoting flowering. PHYTOCHROME INTERACTING FACTOR 4 (PIF4), a protein involved in light signaling, has also been shown to participate in the H2A.Z-mediated regulating of flowering at high temperatures (Kumar et al., 2012). It has been shown that, under non-inductive photoperiods, PIF4 binds the chromatin of *FT* and activates its expression at high temperatures. This regulation is controlled at the level of chromatin accessibility to the *FT* promoter. The presence of H2A.Z in the chromatin of *FT* prevents the binding and activation of *FT* by PIF4, and this can only occur when nucleosomes containing H2A.Z are evicted at high temperatures or in an *arp6* background, where H2A.Z is not properly deposited in the *FT* chromatin (Jarillo and Pineiro, 2015).

Two MADS domain transcription factors, FLM and SHORT VEGETATIVE PHASE (SVP), also play a prominent role in the thermosensory regulation of flowering (Pose et al., 2013; Lee et al., 2013). The *svp* mutant shows no sensitivity to temperature changes, indicating that SVP plays a role in this process. It has recently been shown that two splice forms of *FLM*, FLMβ and FLMδ, give rise to alternative versions of the FLM protein that interact differentially with SVP in a temperature-dependent manner: at lower temperatures, FLMβ forms a complex with SVP that binds its putative targets to repress their expression. The targets of the FLMβ-SVP complex include the floral integrators *SOC1* (Lee et al., 2013; Pose et al., 2013), *FT* and *TSF* (Lee et al., 2013). In contrast, at higher temperatures, the FLMδ form is expressed and binds SVP, acting as a dominant negative that sequesters SVP from FLMβ, preventing the formation of the FLMβ-SVP complex and allowing the de-repression of the floral integrators. High temperatures also influence the stability of the SVP protein. In this way, SVP is less stable at higher temperatures, negatively affecting the formation of the FLMβ-SVP complex and the repression of *FT*, *TSF* and *SOC1* (Lee et al., 2013).

FLC has also been proposed as a candidate to regulate flowering in response to ambient temperature. The *flc-3* mutant is insensitive to temperature changes between 23-27ºC (Lee et al., 2013), and its expression is down-regulated at high temperatures (Blazquez et al., 2003). Moreover, FLC also interacts with SVP and binds *FT* and *SOC1* chromatin to repress their expression (Li et al., 2008; Deng et al., 2011)

2.1.1.4. Autonomous pathway

As discussed above, the acceleration of flowering in response to the cold temperatures of winter relies on the repression of *FLC* (Fig. 1). This gene also responds to internal cues. Specifically, there are a number of proteins that have been grouped into the autonomous pathway of flowering that also converge in the regulation of the floral repressor *FLC* (Fig. 1). This pathway represses *FLC* expression and encompasses several loci, including *FCA*, *FY*, *FPA*, *LUMINIDEPENDENS* (*LD*), *FLOWERING LOCUS D* (*FLD*), *FVE*, *FLOWERING LOCUS K HOMOLOGY DOMAIN* (*FLK*) and *RELATIVE OF EARLY FLOWERING 6*/*JUMONJI 12* (*REF6*/*JMJ12*). Mutants in these loci show a late flowering phenotype and high levels of *FLC* both in LD and SD conditions, and respond to vernalization treatments (Amasino, 2010).

Based on their molecular function, the members of the autonomous pathway can be divided in two groups: they modulate *FLC* expression by either participating in RNA processing or in chromatin remodeling processes. *LD*, *FCA*, *FLK*, *FPA* and *FY* encode proteins with RNA-binding domains (Chan and Struhl, 1997; Baurle and Dean, 2006) and have been proposed to play a role in the silencing of *FLC* by long non-coding RNAs. *FLD*, *FVE*, and *REF6* are among the members of the autonomous pathway that encode chromatin remodeling proteins. *FLD* encodes a homolog of human *LYSINE-SPECIFIC DEMETHYLASE 1* (*LSD1*), and together with another two homologs, *LSD1-LIKE 1* (*LDL1*) and *LDL2*, represses *FLC* expression by decreasing H3K4 methylation levels (Jiang et al., 2007). *FVE* encodes a protein that participates in histone deacetylase complexes (together with HDA6, HDA5 and FLD) that act on *FLC* (Ausin et al., 2004; Gu et al., 2011; Yu et al., 2011; He, 2012; Luo et al., 2015). *REF6* encodes a histone H3K27 demethylase (Lu et al., 2011) that regulates the expression of the floral integrators *FT* and

SOC1 by modulating H3K27 methylation levels (Noh et al., 2004; Lu et al., 2011). The autonomous pathway of flowering does not seem to be linear, but rather an array of genes involved in the repression of gene expression and the establishment of *FLC* basal levels (Amasino, 2010).

2.1.1.5. Gibberellin

Gibberellins (gibberellic acid, GA) are phytohormones that regulate many aspects of plant development, including the activation of the floral transition (Srikanth and Schmid, 2011) (Fig. 1). Bioactive GAs include GA1, GA3, GA4 and GA7, and they regulate gene expression by promoting the degradation of the DELLA proteins, which releases transcription factors that in turn activate the GA-responsive genes (Daviere and Achard, 2013). The role of GA in promoting flowering has been classically ascribed to SD photoperiods. Mutants affected in the biosynthesis of GA display a late flowering phenotype in SD. For instance, the *ga1-3* mutant, which is completely deficient in GA is unable to flower under SD unless supplemented with exogenous GA (Wilson et al., 1992). Also, mutations that cause a constitutive activation of the GA signaling pathway, like *spindly* (*spy*), accelerate flowering (Jacobsen and Olszewski, 1993). GAs promote flowering by activating the expression of *SOC1* and *LFY* in the SAM under SD photoperiods (Blazquez and Weigel, 2000; Moon et al., 2003a; Mutasa-Gottgens et al., 2009) (Fig. 1). However, recent studies have proposed a role for GAs in the activation of flowering also under LD conditions (Porri et al., 2012; Yu et al., 2012; Galvao et al., 2012). In LD, GAs promote flowering by activating the expression of *FT* and its homolog *TSF* in leaves (Porri et al., 2012; Galvao et al., 2012) and also by activating the expression of the *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* genes (*SPLs*) in leaves and in the SAM (Galvao et al., 2012; Yu et al., 2012) (Fig. 1). A recent report unveils a role for GA signaling also in the ambient temperature-mediated induction of flowering (Galvao et al., 2015).

2.1.1.6. Age

Flowering is controlled by the age of the plant through a regulatory network involving the microRNAs miR156 and miR172, and the SPL proteins. SPLs positively regulate the transition from juvenile to adult phases, as well as the floral transition, by activating the expression of the floral integrators *SOC1* and *LFY*, and the meristem identity genes *AP1* and *FUL* (Wang et al., 2009; Yamaguchi et al., 2009) (Fig. 1). The *SPL* genes are targets of miR156, which down-regulates their expression through transcript cleavage (Gandikota et al., 2007). The expression of *miR156* decreases with age, showing an opposite pattern to *miR172*, whose expression levels increase with age and promotes flowering by repressing the expression of *TARGET OF EAT 1*, *2* and *3* (*TOE1*, *2* and *3*), *SCHALAFMÜTZE* (*SMZ*) and *SCHNARCHZAPFEN* (*SNZ*), all repressors of *FT* (Aukerman and Sakai, 2003; Mathieu et al., 2009; Yant et al., 2009) (Fig. 1). The expression of *miR172* is in turn activated by SPL9 in leaves (Wu et al., 2009a). This age-related pathway is also connected with the photoperiod pathway, since GI regulates the expression of *miR172* independently of CO (Jung et al., 2007), and also with the gibberellin pathway, since the DELLAs repress the expression of the SPLs that- activate miR172 in leaves, and *SOC1* and *FUL* at the SAM (Fig. 1). Lastly, apart from their role in the age-related pathway, the opposite expression profiles of miR156 and miR172 at 16ºC and 23ºC indicate the miR156-

miR172 system also plays a role in the thermosensory pathway of flowering (Lee et al., 2010a; Kim et al., 2012). This supports the idea that flowering is controlled by complex regulatory networks rather than linear genetic pathways.

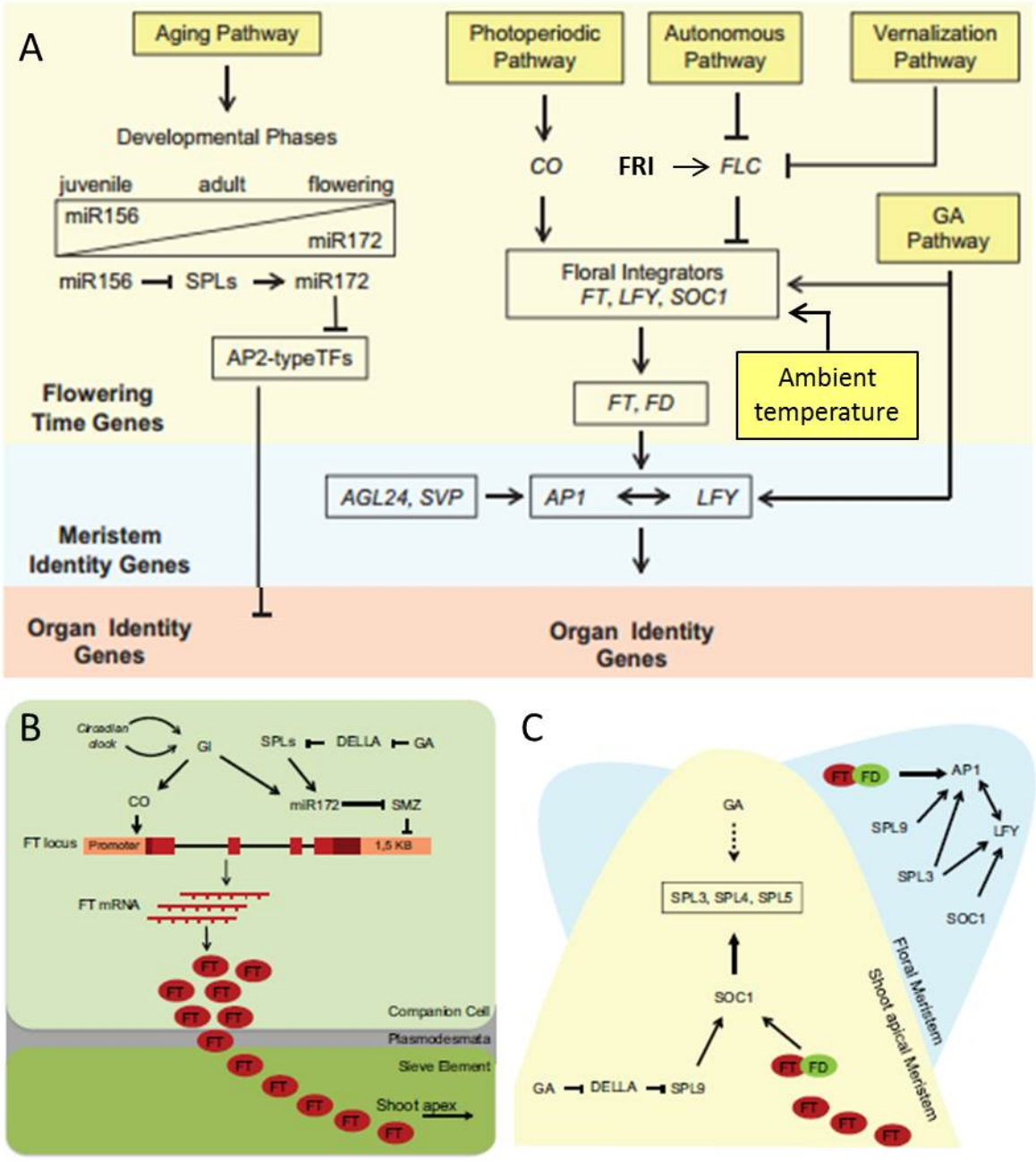

Figure 1. General overview of flowering time regulation in *Arabidopsis thaliana*. (A) Representation of gene regulatory networks that control the floral transition**. (B)** Regulation of *FT* expression in the leaf. **(C)** Activation of the floral meristem identity genes in the SAM by FT. Adapted from Pajoro et al, 2014.

2.1.2. The floral integrators

The complex regulatory network that controls flowering converges in the regulation of a few master genes that will eventually determine the precise moment when the floral transition will take place (Lee and Lee, 2010). These genes are *FT*, *TSF*, *SOC1*, *LFY* and *AGL24*,

named as floral integrators. Their expression is modulated by the balance of both the activating and repressing activities that control the initiation of flowering.

2.1.2.1. The floral integrator FT and its homolog TSF

The regulation of *FT* is a very important point of convergence for all the flowering promoting pathways. In fact, *FT* integrates signals from the photoperiod, vernalization, autonomous, ambient temperature, gibberellin and age-dependent pathways. Therefore, the expression of this gene is finely regulated both in space and time (Andres and Coupland, 2012). The FT protein is part of the florigen and acts as a switch for flowering initiation (Andres and Coupland, 2012) (Fig. 1).

FT gene has a promoter region of 5.7 Kb that contains all the regulatory elements required for its photoperiodic activation (Adrian et al., 2010). Many proteins have been shown to bind *FT* and activate its expression. In LD, CO activates *FT* by binding its transcriptional start site (Andres and Coupland, 2012). Other members of the photoperiod pathway that activate *FT* directly are *GI* and *FKF1* (Sawa and Kay, 2011; Song et al., 2012). CRYPTOCHROME-INTERACTING BASIC HELIX-LOOP-HELIX 1 (CIB1) binds *FT* promoter and activates its expression in response to blue light (Liu et al., 2008b). *FT* expression also responds to increases in ambient temperature through H2A.Z remodeling and the activity of PIF4 (Kumar and Wigge, 2010; Kumar et al., 2012; He, 2012).

Several repressors negatively regulate the expression of *FT* also by binding directly the promoter region and/or the first intron of this gene. This is the case of CDF1, SVP and FLC (Helliwell et al., 2006; Searle et al., 2006; Lee et al., 2007; Song et al., 2012). Other repressors of *FT* include the AP2-like transcription factors TEMPRANILLO 1 and 2 (TEM1 and 2). The balance between CO and TEM is an important mechanism to control *FT* expression in LD (Castillejo and Pelaz, 2008). Other AP2-like proteins such as AP2, TOE1, TOE2, SNZ and SMZ also repress *FT* expression (Kim et al., 2006; Yant et al., 2010; Mathieu et al., 2009; Srikanth and Schmid, 2011; Wang et al., 2011).

In Arabidopsis plants grown under LD conditions, *FT* activity is mainly regulated by the photoperiod. In these conditions, *FT* expression peaks at the end of the day (Zeitgeber time 16, ZT16), and the levels of expression at this time point are higher in LD than on SD (Yamaguchi et al., 2005). *FT* is expressed in the vascular tissue of the leaves (Andres and Coupland, 2012), and the FT protein produced there translocates through the phloem to the SAM, where it activates flowering (Andres and Coupland, 2012; Corbesier and Coupland, 2006) (Fig. 1). In the SAM, the FT protein binds FD to form a complex that will activate an array of genes involved in the promotion of flowering, like *SOC1* and the *SPLs* (Jung et al., 2012; Porri et al., 2012; Torti et al., 2012), which in turn will activate the floral meristem identity genes *FUL*, *LFY* and *AP1* to promote flowering (Wang et al., 2011) (Fig. 1).

In addition to the transcription factor-dependent regulation of *FT* described above, this floral integrator gene is subjected to extensive chromatin-mediated control. Several chromatin-related proteins have been shown to regulate its expression, such as the SWR1 complex, the Polycomb Repressive Complex 2 (PRC2) complex, the Polycomb Repressive Complex 1 (PRC1) protein LIKE HETROCHROMATIN PROTEIN 1 (LHP1), EARLY BOLTING IN

SHORT DAYS (EBS) and REF6. The chromatin regulation of *FT* will be discussed in detail later in the text.

TSF is a close homolog of *FT* and both proteins act redundantly to regulate flowering (Yamaguchi et al, 2005). *FT* and *TSF* are expressed in the leaves and follow the same daily expression patterns (Andres and Coupland, 2012). Both genes are regulated by *CO* and *FLC* and have redundant functions in the phloem to promote flowering through a GA-dependent mechanism (Porri et al., 2012). Despite these similarities, the overexpression of *TSF* leads to an early flowering phenotype that is independent of the photoperiod and the regulation by *FLC* or *CO*, indicating that *TSF* has a distinct role in the promotion of flowering in SD (Yamaguchi et al., 2005; Hiraoka et al., 2013).

2.1.2.2. The floral integrator SOC1

SOC1 encodes a MADS box transcription factor whose expression is regulated by all flowering promoting pathways, either directly by the GA and the age-dependent pathway, or indirectly through *FT* or *FLC* (Lee and Lee, 2010).

Under inductive photoperiods, *CO* positively regulates *SOC1* through *FT* (Lee and Lee, 2010). The complex FT-FD activates *SOC1* expression in the meristem, and the SOC1 protein binds AGL24. Both factors are involved in a positive feedback loop that activates each other's transcription and also *LFY* expression (Lee et al., 2008; Liu et al., 2008a). *SPL3*, *SPL4* and *SPL5* are direct targets of *SOC1* and the FT-FD complex (Jung et al., 2012) (Fig. 1). SPL9 and FUL also activate *SOC1* directly (Wang et al., 2009; Balanza et al., 2014) (Fig. 1). Therefore, the interactions between *SOC1*, *AGL24*, *SPLs* and the floral meristem identity genes promote flowering in LD (Srikanth and Schmid, 2011; Porri et al., 2012).

The expression of *SOC1* is also regulated at the chromatin level. MULTICOPY SUPPRESSOR OF IRA 1 (MSI1) is required for normal *SOC1* expression and for normal H3K4me2 and H3K9Ac in the *SOC1* chromatin (Bouveret et al., 2006). SET DOMAIN GROUP 26 (SDG26) binds the chromatin of *SOC1* and is required for appropriate H3K36me3 levels at this locus (Berr et al., 2015). NUCLEAR FACTOR-Y (NF-Y) regulates *SOC1* expression by modulating H3K27 trimethylation levels, partly through the histone demethylase REF6 (Hou et al., 2014). Lastly, the PHD-containing protein *SHORT LIFE* (*SHL*) has been shown to bind the chromatin of *SOC1* and to be required to maintain normal H3 acetylation levels at this locus (Lopez-Gonzalez et al., 2014).

FLC and *SVP* repress the expression of *SOC1* directly in the apical meristem (Helliwell et al., 2006; Searle et al., 2006; Lee et al., 2007; Li et al., 2008). The expression of *SOC1* is also repressed by *AP2* and regulated by the miR156-miR172 pathway, and also by the SPL and AP2 families of transcription factors (Yant et al., 2010).

AGL24 and *LFY* are also considered to be floral integrators. *AGL24* encodes a MADS box transcription factor that acts as a floral activator in a similar way to *SOC1* (Michaels et al., 2003), integrating signals from the photoperiod, vernalization, GA and autonomous pathways. However, unlike *SOC1*, the expression of *AGL24* is not controlled by *FLC* (Lee and Lee, 2010; Srikanth and Schmid, 2011). AGL24 and SOC1 form a heterodimer that activates *LFY* (Lee et al.,

2008; Liu et al., 2008a; Jung et al., 2012). *AGL24* and *SVP* positively regulate *AP1* and *LFY* (Grandi et al., 2012) (Fig. 1). *LFY* was first identified as a regulator of floral meristem identity, but it has been shown to also participate in the floral transition, integrating signals from the autonomous, GA and age-related pathway (Weigel and Nilsson, 1995; Blazquez et al., 1997; Yamaguchi et al., 2009).

3. Epigenetic regulation of flowering

Chromatin remodeling processes play a central role in the establishment of gene expression patterns that drive plant development. Chromatin structure provides a mechanism that ensures the stability of gene expression patterns throughout the mitotic cell divisions that take place in a specific cell line (Jarillo et al., 2009). Numerous studies have highlighted how chromatin dynamics is essential for the correct regulation of the components of the flowering pathways as well as the floral integrators (Farrona et al., 2008; Crevillen and Dean, 2011; Choi et al., 2011; He, 2012) .

Eukaryotic chromatin is formed by basic structural units called nucleosomes. Each nucleosome encompasses a histone octamer (two dimers H2A-H2B and a tetramer H3-H4), and a 140 bp DNA strand wrapped around the histone octamer (Luger et al., 1997). Decondensed chromatin or euchromatin contains most of the genes that are actively transcribed, as its open conformation allows access to the transcription machinery. Highly condensed chromatin or heterochromatin usually contains transcriptionally inactive regions of the genome.

Chromatin remodeling proteins can be divided into three groups:

- Chromatin remodeling complexes that alter the interaction between the DNA and the histone octamer non-covalently in an ATP-dependent manner (Clapier and Cairns, 2009). This is the case of SWI/SNF2 complexes.
- Complexes that catalyze the exchange of canonical histones by histone variants.
- Complexes involved in the post-translational covalent modification of histones and DNA, affecting the condensation status of the chromatin (Altaf et al., 2009).

The combination and crosstalk between covalent modifications of histones has been proposed to constitute a code that sets the basis for transcriptional regulation through chromatin signaling, and adds an additional layer of regulation superimposed on transcription factors regulation (Lee et al., 2010b). Histone acetylation and trimethylation of lysine (K) 4 and 36 of histone H3 (H3K4me3 and H3K36me3) are associated with transcriptionally active states (Carrozza et al., 2003; Rando, 2007; Xu et al., 2008b). These modifications act as platforms for the recruitment of effector proteins that modify the transcriptional status of underlying genes; however, histone acetylation has an additional physical effect on chromatin structure. The addition of a negatively charged acetyl group has been proposed to neutralize the positive charge of K in the histones and contributes to release the interaction of histones with the DNA and to open the chromatin, so it becomes more accessible to the transcriptional machinery (Berr et al., 2011). Acetylation of K in histones from a donor acetyl-CoA is catalyzed by histone acetyltransferases (HATs). Arabidopsis HATs can be classified into four families: GNAT, MYST,

CBP/p300 and TAF1/TAF_{II}250 (Berr et al., 2011). The balance between acetylation/deacetylation is maintained by histone deacetylases (HDACs), which remove the acetyl group from histones. Arabidopsis HDACs can be classified into RPD3/HDA1 superfamily, SIR2 family, and HD2-like family.

Conversely, H3K9me3 and H3K27me3 modifications are associated with the repression of gene expression (Carrozza et al., 2003; He and Amasino, 2005; Ringrose and Paro, 2007). The deposition and maintenance of the repressive mark H2K27me3 involves the activity of Polycomb group (PcG) protein complexes. These complexes can be divided in two groups: Polycomb Repressive Complex 1 and 2 (PRC1 and PRC2). PRC2 comprises four subunits and was first identified in *Drosophila melanogaster*. In this organism, each subunit is encoded by a single gene (Simon and Kingston, 2013). In Arabidopsis, there are several homologs for each subunit, and depending on the subunit combination, three different PRC2 complexes can be found: the EMF, VRN and FIS complexes (Mozgova et al., 2015; Del Prete et al., 2015; Xiao and Wagner, 2015). The PRC2 complex carries the methyltransferase activity that catalyzes the initial step of H3K27 trimethylation. The PRC1 complex was also initially identified in Drosophila and also encompasses four subunits. Although the existence of a plant PRC1 has remained elusive for years, many subunits that share homology to PRC1 components have been identified in Arabidopsis (Merini and Calonje, 2015). PRC1 is recruited to H3K27me3-marked genes by LHP1, which recognizes H3K27me3 (Turck et al., 2007; Zhang et al., 2007; Exner et al., 2009) and is necessary for maintaining gene repression in (Libault et al., 2005; Nakahigashi et al., 2005). In turn, PRC1 catalyzes H2A monoubiquitination (H2Aub), another repressive mark in the chromatin of target genes. In this way, PRC2 and PRC1 complexes cooperate to maintain a transcriptionally repressed state in their target genes. Even though it was proposed that the initial step for PcG repression was the PRC2-mediated methylation of H3K27, followed by the recruitment of PRC1, recent studies are showing that the sequence of events might be the opposite, at least for a subset of genes. The fact that in many genes, PRC1 seems to be required for H3K27me3 deposition by PRC2 and that the genome-wide distribution of PRC1 components does not seem to clearly overlap with the distribution of the H3K27me3 mark has allowed to propose a working model in which PcG repression is initiated by PRC1, followed by the recruitment of PRC2 (Merini and Calonje, 2015).

Several studies have highlighted the importance of chromatin-mediated mechanisms in the regulation of key flowering regulatory genes, including *FT* and *FLC* (He, 2012).

3.1. Epigenetic regulation of FT

Many chromatin modifiers have been shown to be involved in the regulation of the floral integrator *FT* (He, 2012) (Fig. 2). PRC2-mediated repression of *FT* plays an important role in the regulation of this gene. The PRC2 histone methyltransferase CURLY LEAF (CLF) binds *FT* directly and is required for the deposition of H3K27me3 and the transcriptional repression of this locus (Jiang et al., 2008). Other PRC2 components such as *SWINGER* (*SWN*), *EMBRYONIC FLOWER 2* (*EMF2*) and *FERTILIZATION-INDEPENDENT ENDOSPERM* (*FIE*) are also necessary for *FT* repression (Jiang et al, 2008; Farrona et al, 2011) (Jiang et al., 2008; Farrona et al., 2008). As mentioned before, REF6 is a histone demethylase of H3K27 that participates in the

demethylation of H3K27me3 in *FT* and is required for its expression (Lu et al., 2011). This way, the PRC2 and REF6 dynamically control H3K27me3 levels at *FT*.

As previously discussed, PRC1 components recognize and bind H3K27me3 modifications deposited by PRC2. Arabidopsis LHP1, EMF1 and Arabidopsis B lymphoma Moloney murine leukemia virus insertion region1 homolog 1A (BMI1), three PRC1 components, are also required for *FT* repression (Turck et al., 2007; Farrona et al., 2011; Moon et al., 2003b; Bratzel et al., 2010; Pico et al., 2015).

Interestingly, BMI1 PRC1 components participate in the repression of *miR156* (Pico et al., 2015). Conversely, the PRC1 component EMF1 participates in the regulation of *SPLs* and *MIR172* genes. Accordingly, plants impaired in *EMF1* function displayed misexpression of these genes early in development, which contributes to a CONSTANS-independent up-regulation of *FT* leading to the earliest flowering phenotype described in Arabidopsis.

The H3K4 demethylases EARLY FLOWERING 6 (ELF6) and JUMONJI4/14 (JMJ4/JMJ14) play redundant roles in the repression of *FT* expression (Jeong et al., 2009; Yang et al., 2010). JMJ14 directly binds the chromatin of *FT* and the loss of function of this demethylase causes an increase of H3K4me3 and an up-regulation of this floral integrator leading to early flowering of *jmj14* mutant plants (Yang et al., 2010; Lu et al., 2010; Jeong et al., 2009), indicating that the dynamics of the active mark H3K4me3 are also important for *FT* regulation. Loss of JMJ14 activity also leads to a reduction in H3K27me3 (Jeong et al., 2009; Yang et al., 2010), suggesting that there is a crosstalk between both marks at *FT*.

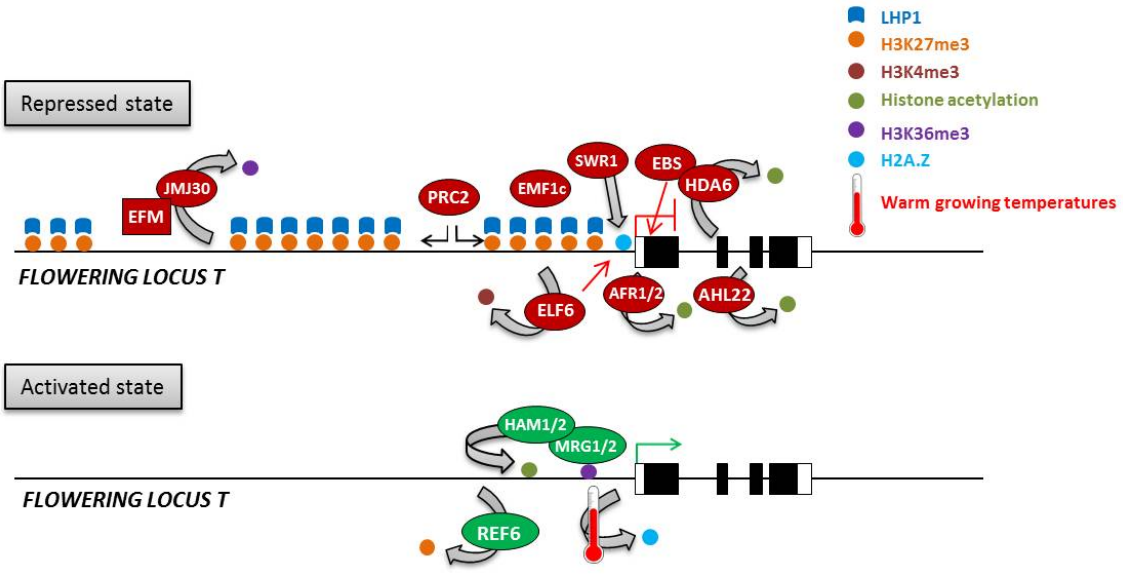

Figure 2. Epigenetic mechanisms regulating the repression and activation of *FT* .

Recent reports suggest the existence of an EMF1-containing PRC1-like complex. This complex contains EMF1, LHP1 and JMJ14, and represses the expression of *FT* directly before dusk and at night by maintaining H3K27me3 levels in this gene, in order to prevent photoperiod-independent activation of flowering (Wang et al., 2014b).

Moreover, the MYB transcription factor EARLY FLOWERING MYB PROTEIN (EFM) plays an important key role in directly repressing *FT* expression in the leaf vasculature (Yan et al., 2014). EFM interacts with the H3K36me2 demethylase JMJ30, which forms a negative feedback regulatory loop with the light-responsive circadian clock, to specifically demethylate H3K36me2 at *FT*.

As mentioned above, H2A.Z is essential in the thermosensory flowering response in Arabidopsis. The SWR1 complex catalyzes the exchange of H2A by this histone variant. Global H2A.Z occupancy studies have shown that *FT* chromatin is enriched in this histone variant, indicating that this floral integrator is a target of the SWR1-C (Zilberman et al., 2008; Kumar and Wigge, 2010). Increases in growing temperatures cause the eviction of nucleosomes containing H2A.Z from *FT* and allow its transcription by RNA Pol II (Kumar and Wigge, 2010). Mutants in SWR1 components such as *arp6* show temperature-insensitive activation of *FT* and early flowering.

3.1.1. Regulation of FT *by histone acetylation*

Recent reports have highlighted the importance of histone acetylation in the regulation of *FT*. Plants overexpressing *CO* show an increase in H3K9K14 acetylation (H3K9K14Ac) in transcribed and regulatory regions of *FT* (Andres and Coupland, 2012), which indicates that the photoperiodic activation of *FT* mediated by CO correlates with increased histone acetylation.

Histone deacetylation also seems to play a role in the regulation of *FT*. *SAP30 FUNCTION-RELATED 1* and *2* (*AFR1* and *2*) are components of an RPD3-like HDAC complex that participates in the photoperiodic deacetylation of *FT* (Gu et al., 2013). These proteins physically interact with HDA19, an RPD3 HDAC, and this complex is required to down-regulate the expression of *FT* at dusk under LD. AFR1 and AFR2 bind the chromatin of *FT* and mediate histone deacetylation in this gene. These proteins might be recruited to *FT* chromatin by two transcription factors, AGL18 and AGL15, and deacetylate the chromatin of *FT* at dusk to prevent premature flowering under LD conditions (Gu et al., 2013).

The AT-hook protein AHL22 also participates in the regulation of *FT* through histone deacetylation (Yun et al., 2012). Plants overexpressing AHL22 display late flowering and reduced levels of *FT* expression that correlate with decreased H3 acetylation in this locus. AHL22 is able to interact with the HDACs HD1, HDA6 and HDA9, and also binds an AT-rich region in the chromatin of *FT*, which suggests that this protein modulates *FT* expression through histone deacetylation.

Recent reports have revealed a role for EBS, a PHD-containing protein that acts as a "reader" of H3K4me3 in the regulation of *FT* (Lopez-Gonzalez et al., 2014). EBS is required to repress the expression of *FT* (Gomez-Mena et al., 2001; Pineiro et al., 2003). López-González et al. have shown that EBS recognizes H3K4me2/3 and binds regulatory regions in the chromatin of *FT*. *EBS* is required to maintain low levels of H3 acetylation at *FT*, and the fact that the EBS protein physically interacts with HDA6 suggests that the interaction between EBS and HDAC

complexes contributes to maintain *FT* chromatin in an inactive state and prevent precocious activation of flowering.

3.2. Epigenetic regulation of FLC

FLC represses the expression of the floral integrators *FT* and *SOC1* and therefore plays a fundamental role in flowering regulation. This gene has become a paradigm for the study of chromatin modifications and their relationship with the transcription machinery (Berr et al., 2011; Crevillen and Dean, 2011). The epigenetic regulation of *FLC* is highly complex and involves a large number of chromatin-related proteins. Many chromatin proteins have been shown to play a role in the establishment of high expression levels of *FLC* in the early stages of Arabidopsis that prevent a premature activation of flowering (Fig. 3).

The monoubiquitination of K123 of histone H2B (H2Bub1) is associated with activation of gene expression (Wood et al., 2003b). In yeast, a complex formed by the E2 ubiquitin-conjugating enzyme RAD6 and the E3 ubiquitin ligase BRE1 participates in H2B monoubiquitination at specific loci (Wood et al., 2003a). Three homologs of *RAD6* are present in Arabidopsis, namely *UBIQUITIN CONJUGATING ENZYME 1* (*UBC1*), *UBC2* and *UBC3*, as well as two homologs of *BRE1*, namely *HISTONE MONOUBIQUITINATION 1* (*HUB1*) and *HUB2* (Fleury et al., 2007; Xu et al., 2009; Liu et al., 2007). It has been proposed that UBC1 and UBC2 function redundantly and, together with the tetramer formed by two molecules of HUB1 and two of HUB2, they participate in the monoubiquitination of H2B, which is necessary to initiate *FLC* transcription (Berr et al., 2011).

Other histone marks typically associated with the transcriptionally active state of *FLC* are H3K4me3 and H3K36me3 (He and Amasino, 2005) (Fig. 3). Some of the proteins involved in the deposition of these marks in the chromatin belong to the Trithorax group of proteins (TrxG) (Liu et al., 2010). Some members of this family include *ARABIDOPSIS TRITHORAX LIKE1* (*ATX1*), *ATX2*, *SET DOMAIN GROUP2*/*ARABIDOPSIS TRITHORAX-LIKE RELATED3* (*SDG2*/*ATXR3*) and *SDG25*/*ATXR7*, which mediate H3K4 trimethylation, and *SDG8*/*EARLY FLOWERING IN SHORT DAYS* (*EFS*), involved in H3K36 trimethylation in the chromatin of *FLC*, in the context of the COMPASS-like complex in Arabidopsis (Cartagena et al., 2008; Thorstensen et al., 2008; Berr et al., 2009; Tamada et al., 2009; He, 2012). Mutations in *ATX1* originate an early flowering phenotype, as well as defects in the leaves and in plant resistance to pathogens (Alvarez-Venegas et al., 2003; Alvarez-Venegas and Avramova, 2005). Mutations in *SDG8/EFS* locus, which encodes the main methyltransferase of H3K36 in Arabidopsis, cause an acceleration of flowering and pleiotropic phenotypic alterations (Cazzonelli et al., 2009). This protein bears a SET domain in its carboxy terminus and a CW domain in the amino terminus, which recognizes different states of methylation of H3K4, preferably mono- and dimethylated (Hoppmann et al., 2011). *SDG8/EFS* is required for the deposition of specific H3K36me2/3 marks and to activate the expression of *FLC* and the *MAF* genes (Xu et al., 2008b).

In yeast, the RNA polymerase II (Pol II) Associated Factor 1 (PAF1) complex associates with RNA polymerase II and promotes transcription through the recruitment of the H3K4 methyltransferase SET1, and the H3K36 methyltransferase SET2, which are part of the COMPASS complex (Berr et al., 2011). A similar mechanism seems to be conserved in

Arabidopsis, since mutants affected in some of the described PAF1 subunits display an acceleration of flowering and a reduction of *FLC* expression which is directly related to a decrease in H3K4me3 and H3K36me3 in this locus (Yu and Michaels, 2010). In fact, homologs of components of both complexes have been identified in Arabidopsis. Among the Arabidopsis homologs of PAF1-C components have been found *VIP2*/*ELF7*, *VIP3*, *VIP4*, *VIP5*, *VIP6*/*ELF8* and *PHP*/*CDC73* (Park et al., 2010; Yu and Michaels, 2010). The Arabidopsis COMPASS-C comprises ATX1, ATX2, ATXR7, WDR5 HOMOLOG A (WDR5a), RbBP5 LIKE (RBL), ARABIDOPSIS Ash2 RELATIVE (ASH2R) and SDG8/EFS (Jiang et al., 2011; Kim and Sung, 2012). All these subunits are necessary for proper expression of *FLC* and the *MAF* genes (Zhang et al., 2003; He et al., 2004). Thus, Arabidopsis PAF1-C could recruit SDG8, a component of COMPASS-C, allowing the coordinated participation of both complexes in the transcriptional activation of *FLC* through the increase in H3K4 and H3K36 methylation, prior to the vernalization process (Krogan et al., 2003a; Kim and Sung, 2012). It has recently been shown that *SDG8* has a dual role in the activation of *FLC*; it acts as a histone methyltransferase and also recruits a FRI-containing complex that activates *FLC* (Ko et al., 2010; Choi et al., 2011).

All these epigenetic mechanisms just described contribute to activate *FLC* during the early stages of development. As previously mentioned, the establishment of elevated levels of *FLC* is essential for the vernalization requirement, and *FRI* plays an important role in this process. FRI activates *FLC* transcription through a co-transcriptional mechanism that involves the interaction with the nuclear cap-binding complex (Geraldo et al., 2009; Crevillen et al., 2013), and it also acts as a scaffold protein that mediates the recruitment of several chromatin remodeling activities required for the activation of *FLC* (Choi et al., 2011).

Vernalization relies in the stable epigenetic silencing of *FLC*, which is mediated by the deposition of repressive marks like H3K27me3 and H3K9me3 in the chromatin of this gene (Fig. 3). Initial studies showed that the VEL family members *VIN3* and *VRN5* are required for a proper vernalization response and to establish the repressed state in the chromatin of *FLC* through histone deacetylation and the incorporation of methylated H3K9 and H3K27. *VIN3* is expressed upon cold exposure (Sung and Amasino, 2004), while *VRN5* is constitutively expressed, and both proteins have been shown to interact during the course of vernalization (Greb et al., 2007). Exposure to cold temperatures triggers the association of the PHD-containing proteins VIN3, VRN5 and VIL1 with the PRC2 component VRN2 and the formation of a PHD-PRC2 complex (De Lucia et al., 2008). This complex also contains CLF, FIE and MSI1 (Wood et al., 2006). At the beginning of the cold exposure, VRN5 directs the PHD-PRC2 complex to a discrete region of *FLC* intron 1, where it starts to catalyze the deposition of H3K27me3 and initiates silencing (De Lucia et al., 2008). The amount of H3K27me3 locally deposited correlates with the duration of the cold exposure (Song et al., 2013). After the plants have returned to warm conditions, VRN5 localizes more broadly and the PHD-PRC2 complex (without VIN3) spreads across *FLC*, resulting in an increase of H3K27me3 which is also correlated with the length of the cold treatment (De Lucia et al., 2008; Angel et al., 2011; Finnegan and Dennis, 2007) (Fig. 3). The interaction between the PHD proteins and PRC2 enhances the activity of PRC2 at *FLC* and contributes to the increase of H3K27me3 and the silencing of the gene. More recent studies show that all members of the VEL family mediate the vernalization response in Arabidopsis by repressing the expression of *FLC* and the *MAF*

genes, but they have different functions and targets among the *FLC* family (Kim and Sung, 2013).

The H3K27me3 mark is recognized by LHP1, a component of the Arabidopsis PRC1 (Zhang et al., 2007; Turck et al., 2007). LHP1 binds this epigenetic modification through its chromodomain and is required to maintain a silenced state of *FLC* (Sung et al., 2006; Mylne et al., 2006). Another putative member of PRC1, VRN1, a protein with two B3 domains, could function together with LHP1 to maintain the silenced state of *FLC* after the cold (Zheng and Chen, 2011; Turck et al., 2007). After PRC2 has depositedH3K27me3 repressive mark in the *FLC* chromatin and the cold stimulus has ceased, PRC1 would be responsible for reading the silenced state and locking *FLC* chromatin to avoid the reactivation of the gene once temperatures get warmer in the spring and plants acquired flowering competence (He, 2012).

Recent work has shown that vernalization induces the expression of the *FLC* long non-coding RNAs (lncRNAs) *COLD INDUCED LONG ANTISENSE INTRAGENIC RNA* (*COOLAIR*) and *COLD ASSISTED INTRONIC NONCODING RNA* (*COLDAIR*) (Swiezewski et al., 2009; Heo and Sung, 2011; Ietswaart et al., 2012). COLDAIR interacts with CLF and is required for the recruitment of PRC2 to the *FLC* chromatin to initiate its epigenetic silencing. In contrast, COOLAIR seems to participate in silencing *FLC* in response to vernalization independently of PRC2 (Berry and Dean, 2015; Csorba et al., 2014).

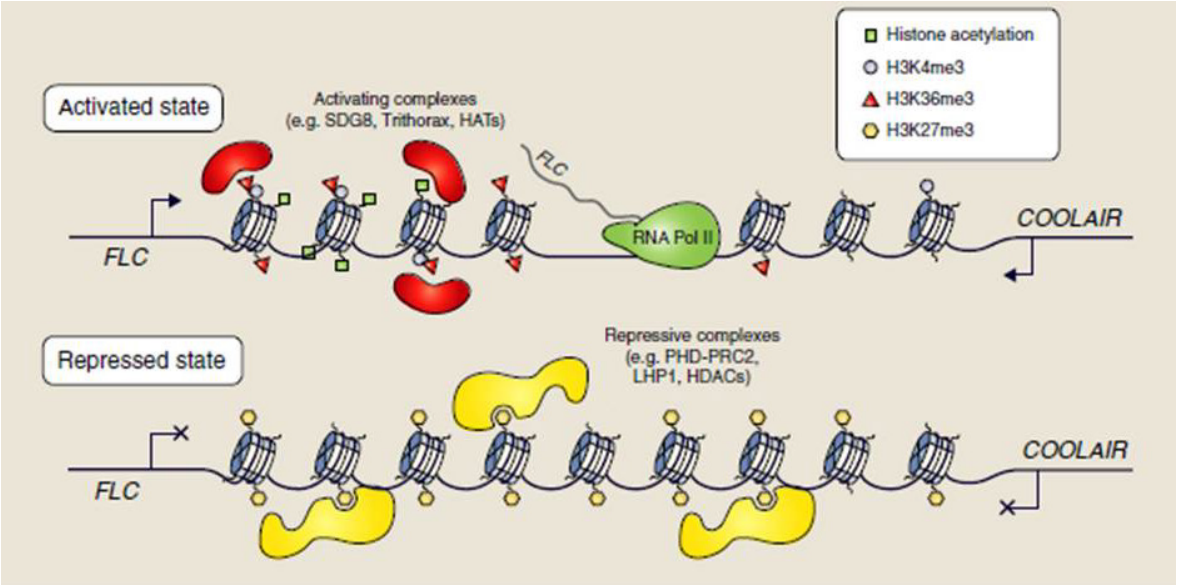

Figure 3. Epigenetic mechanisms operating in the activation and repression of *FLC*. Adapted from Berry and Dean, 2015.

After the plant has flowered, *FLC* expression has to be reset to ensure that Arabidopsis plants in the next generation recover a vernalization requirement (Berry and Dean, 2015). This involves the removal of the repressive marks from *FLC* chromatin. A recent study has revealed a role for *ELF6* in the resetting of *FLC* expression (Crevillen et al., 2014). In addition to the H3K4 demethylase activity proposed initially, ELF6 also functions as a H3K27me3 demethylase. *elf6* mutants fail to remove the H3K27me3 mark induced by vernalization and inherit a partially vernalized state, showing lower *FLC* levels in the next generation (Crevillen et al., 2014).

3.2.1. *Regulation of* FLC *by histone acetylation*

In light of the extensive epigenetic regulation of *FLC*, not surprisingly, histone acetylation also controls the expression of this gene. Several HAT and HDAC proteins have been shown to play a role in the regulation of *FLC*.

A number of studies showed that two components of the autonomous pathway, *FVE* and *FLD*, regulate the acetylation levels at *FLC* (He et al, 2003; Ausín et al, 2004; Kim et al, 2004) (He et al., 2003; Ausin et al., 2004). Vernalization also regulates *FLC* through histone acetylation and methylation (Bastow et al., 2004; Finnegan et al., 2004; He et al., 2004; Sung and Amasino, 2004; Amasino, 2005; He and Amasino, 2005). Recent work has shown that FLD and FVE (or MSI5, an FVE homolog playing partially redundant roles with FVE) interact with HDA6 to form a complex that represses *FLC* (Yu et al., 2011; Gu et al., 2011). HDA5 has also been shown to interact with HDA6, FVE and FLD, suggesting that it could also be part of a deacetylase complex that represses *FLC*. This is experimentally supported by the late flowering phenotype and increased expression of *FLC* displayed by *hda5* mutants (Luo et al., 2015). Thus, histone acetylation is an important modification that regulates *FLC* expression (Fig. 3).

3.2.2. *Regulation of* FLC *by SWR1-mediated incorporation of H2A.Z*

As mentioned above, SWR1-C is required for transcriptional activation of *FLC* (Noh and Amasino, 2003; Choi et al., 2005; Choi et al., 2007; Deal et al., 2005; Deal et al., 2007; March-Diaz et al., 2007; March-Diaz et al., 2008; Lazaro et al., 2008). Yeast SWR1 is a multiprotein complex that catalyzes the exchange of H2A by the histone variant H2A.Z. SWR1-C uses the energy from the hydrolysis of ATP to mediate the exchange of H2A by H2A.Z and destabilize the nucleosome structure (Morrison and Shen, 2009).

Yeast SWR1-C comprises 14 subunits (Wu et al., 2009b; Nguyen et al., 2013) (Fig. 4). The incorporation of H2A.Z *in vivo* depends only on some of those subunits, namely Swr1, Swc2, Arp6, Swc6 and Yaf9 (Krogan et al., 2003b; Zhang et al., 2004). The role of H2A.Z in the regulation of gene expression has remained controversial for years (Jarillo and Pineiro, 2015). This histone variant is enriched in the TSS of genes (Raisner et al., 2005; Zilberman et al., 2008). It has been proposed that nucleosomes containing H2A.Z in this position might poise genes for transcriptional activation (Fan et al., 2002). However, H2A.Z in gene bodies negatively correlates with transcription and is associated with gene silencing (Cui et al., 2009; Kumar and Wigge, 2010; Farris et al., 2005; Barski et al., 2007; Jin et al., 2009a). Therefore, H2A.Z might perform opposite roles in the regulation of gene expression depending on its position within the genes.

Homologues of yeast SWR1 have been identified and characterized in Arabidopsis. *PHOTOPERIOD INDEPENDENT EARLY FLOWERING 1* (*PIE1*) encodes the homologue of yeast Swr1, the ATPase catalytic subunit of the complex, which also acts as a scaffold for the assembly of the rest of SWR1 components and binds H2A.Z (Wu et al., 2005; March-Diaz et al., 2007; Choi et al., 2007). PIE1 also interacts with ARP6 and SWR1 COMPLEX 6 (SWC6), while ARP6 can also interact with SWC6 (Choi et al., 2007; March-Diaz et al., 2007; Lazaro et al., 2008). Other SWR1 components conserved in Arabidopsis include ACTIN-RELATED PROTEIN 4

(ARP4) and YEAST AF9 (YAF9). These observations strongly support the existence of a SWR1-C in Arabidopsis. Consistent with this, the Arabidopsis genome contains three genes that encode H2A.Z proteins, *HTA8*, *HTA9* and *HTA11* (Yi et al., 2006).

The genetic and molecular characterization of *PIE1*, *ARP6* and *SWC6* indicate that these genes have a role in the regulation of flowering time (Noh and Amasino, 2003; Choi et al., 2005; Choi et al., 2007; Deal et al., 2005; Deal et al., 2007; Lazaro et al., 2008; Martin-Trillo et al., 2006; March-Diaz et al., 2007; March-Diaz et al., 2008). Mutants in these three genes show an early flowering phenotype and similar developmental defects. Also, the expression of *FLC*, *MAF4* and *MAF5* is reduced in these mutants, and this is accompanied by an increased expression of *FT* and *SOC1*. SWC6 and ARP6 bind *FLC* chromatin directly (Choi et al., 2007), and PIE1 and ARP6 are required for normal H2A.Z levels in the 5' and 3' ends of the *FLC*, *MAF4* and *MAF5* loci and their transcriptional activation (Deal et al., 2007). These data indicate that H2A.Z deposition mediated by SWR1 is necessary for the activation of *FLC* and therefore plays an important role in the control of flowering in Arabidopsis. Consistent with these observations, *hta9 hta11* double mutants display phenotypic alterations that resemble those of SWR1 mutants and an early flowering phenotype that correlates with reduced levels of *FLC* (March-Diaz et al., 2008). The phenotype of *hta8 hta9 hta11* (*h2a.z*) triple mutants also resembles that of SWR1 mutants; however, the phenotypic differences between *pie1* and *h2a.z* mutants, and the fact that *pie1 h2a.z* mutants are lethal, indicates that SWR1 also performs independent functions from H2A.Z (Coleman-Derr and Zilberman, 2012).

3.2.3. Functional interplay between SWR1 and NuA4 complexes

Some of the SWR1-C subunits are also present in other chromatin remodeling complexes. This is the case of yeast Yaf9, Swc4, Act1 and Arp4, which are also part of the NuA4 complex (NuA4-C) (Kobor et al., 2004; Owen-Hughes and Bruno, 2004; Raisner and Madhani, 2006; Chittuluru et al., 2011; Billon and Cote, 2013) (Fig. 4). Yeast NuA4-C is a 13-subunit complex that shows HAT activity for histones H4, H2A and H2A.Z (Lu et al., 2009) (Fig. 4), and it has been shown to cooperate with NuA4 in the H2A.Z-dependent gene activation (Lu et al., 2009). Interestingly, acetylation of H2A and H4 by NuA4 stimulates H2A.Z incorporation by SWR1 (Altaf et al., 2010). A mechanistic model for the interplay between these two complexes has been proposed in yeast. According to this working model, NuA4 acetylates H4, and this facilitates the recruitment of SWR1 and subsequent exchange of H2A by H2A.Z in the regions where H4 has been acetylated. Then, the newly incorporated H2A.Z is in turn acetylated by NuA4 (Babiarz et al., 2006; Keogh et al., 2006; Millar et al., 2006; Valdes-Mora et al., 2012). This model is supported by the presence of shared subunits by the two complexes and by the fact that in humans, both complexes seem to have been merged into a single complex called p400/TIP60, which carries both enzymatic activities, H2A exchange and histone acetylation (Choi et al., 2009). Most of the subunits of the yeast NuA4-C are conserved in Arabidopsis, which supports the notion that this complex could also be preserved in plants.

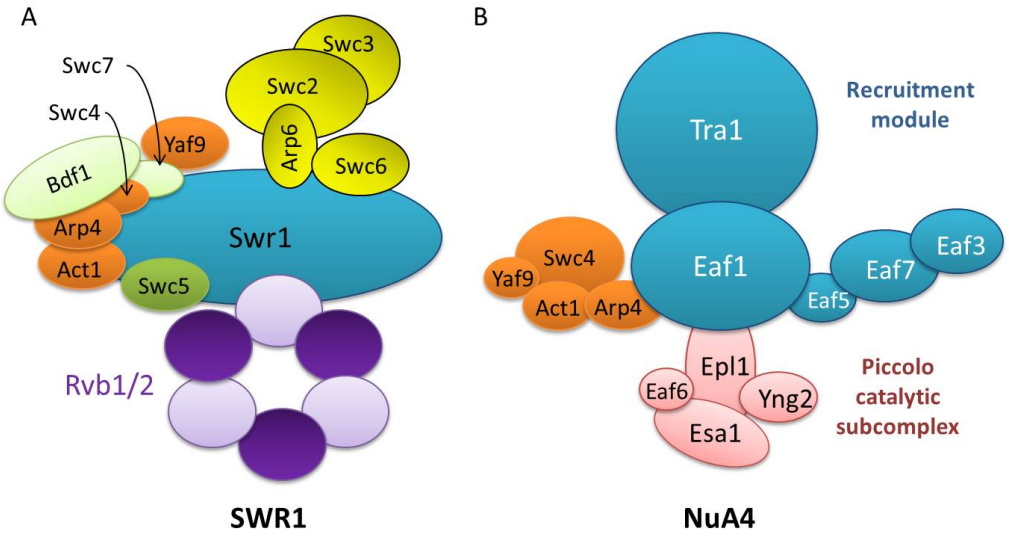

Figure 4. Representation of *S. cerevisiae* SWR1 (A) and NuA4 (B) complexes. Depicted subunit arrangement is based on previous studies. Subunits shared by the two complexes (Act1, Arp4, Swc4 and Yaf9) are represented in orange. Adapted from Nguyen et al, 2013 and Chittuluru et al, 2012.

3.3. The NuA4 complex in the regulation of the floral transition in Arabidopsis

The NuA4-C was initially identified and purified in yeast. It comprises the following subunits: the catalytic subunit Esa1, and Epl1, Yng2, Eaf6, Tra1, Eaf1, Eaf5, Eaf7, Eaf3, Arp4, Act1, Yaf9 and Swc4. NuA4 can be found in two forms, the Piccolo NuA4 subcomplex and the large NuA4 complex. The Piccolo subcomplex is composed by the tetramer Esa1, Yng2, Epl1 and Eaf6 and is responsible for the global non-targeted histone acetylation of chromatin (Boudreault et al., 2003), whereas the large 13-subunit NuA4-C accounts for the targeted histone acetylation at specific loci (Auger et al., 2008; Brown et al., 2001; Mitchell et al., 2008; Ginsburg et al., 2009). NuA4 plays important roles in transcriptional regulation, heterochromatin silencing, cell cycle progression and DNA repair, and it is highly conserved in eukaryotic organisms (Doyon and Cote, 2004; Auger et al., 2008).

A number of recent reports have described the role of several subunits of the putative Arabidopsis NuA4-C in the regulation of flowering time and in particular, in the acetylation-mediated control of *FLC* expression. Among the NuA4-C subunits shared with SWR1, the homologues of Arp4 and Yaf9 have been shown to regulate flowering time in Arabidopsis. Plants with reduced expression of *ARP4* show early flowering under LD and phenotypic alterations that resemble those of other SWR1 subunits (Kandasamy et al., 2005). Arabidopsis contains two genes that share homology with yeast *Yaf9*, named as *YAF9A* and *YAF9B*/*TAF14* (Choi et al., 2011). *YAF9A* has also been shown to participate in the regulation of flowering by modulating the expression of *FLC* and *FT* (Zacharaki et al., 2012). Mutations in *YAF9A* cause an early flowering phenotype that correlates with decreased expression of *FLC* and increased *FT*. These mutants present reduced H4 acetylation levels in *FLC* and increased in *FT* chromatin, indicating that *YAF9A* participates in regulating H4 acetylation levels in these genes. Also, *yaf9a yaf9b* double mutant plants display more severe phenotypic alterations than the single

mutants, including reduced plant size and altered morphology (Choi et al., 2011). In fact, *yaf9a yaf9b* plants flowered significantly earlier than single mutants under both LD and SD conditions (Bieluszewski et al., 2015), demonstrating that there is certain degree of redundancy between both genes in the regulation of flowering.

The Arabidopsis homologues of *Esa1* are *HISTONE ACETYLTRANSFERASE OF THE MYST FAMILY 1* and *2* (*HAM1* and *HAM2*). They encode the two MYST family members of HATs in Arabidopsis and are functionally redundant (Latrasse et al., 2008). While *ham1* and *ham2* single mutants did not display any phenotypic alteration, the *ham1 ham2* double mutant combination resulted to be lethal, causing severe defects in gametophyte development and arrest in mitotic cell cycle in the early stages of gametogenesis (Latrasse et al., 2008). Given the unfeasibility to use *ham1 ham2* double mutants to analyze the function of these proteins in flowering, Xiao et al used artificial micro RNA (amiRNA) and overexpression approaches to unveil the functions of these proteins overcoming functional redundancy and lethality effects (Xiao et al., 2013). Plants that overexpress *HAM1* show delayed flowering, increased *FLC* and *MAF3*/*4* expression and decreased *SOC1*. Conversely, plants with silenced *HAM1*/*2* display an early flowering phenotype, decreased *FLC* and *MAF3*/*4*, and increased *SOC1* expression. Moreover, mutations in *FLC* almost fully suppress the late flowering phenotype caused by the overexpression of *HAM1*, indicating that the effect of *HAM1* depends on *FLC*. Lastly, ChIP analyses performed with *HAM1* overexpression lines and *HAM1*/*2* amiRNA lines showed that HAM1 and HAM2 affect the levels of H4K5Ac on *FLC* and *MAF3*/*4*. These results indicate that these proteins are required for the repression of flowering through the modulation of H4K5Ac levels and transcriptional activation of *FLC*, *MAF3* and *MAF4*.

A recent study has shown that HAM1 and HAM2 interact with MORF4-RELATED GENE1 and 2 (MRG1 and 2), the Arabidopsis homologues of yeast Eaf3 (Xu et al., 2014). These proteins bind and recognize H3K36me3, and therefore might act as “readers” of this mark (Bu et al., 2014). *MRG1* and *MRG2* are required for the photoperiodic activation of *FT* as they interact with CO to activate the expression of *FT* under LD (Bu et al., 2014). These proteins bind the chromatin of *FT* and promote H4 acetylation in this locus (Xu et al., 2014).

In parallel, ESA ASSOCIATED FACTOR 1 (EAF1) has been characerized as a novel NuA4 subunit in Arabidopsis, being encoded by two almost identical genes (*EAF1A* and *EAF1B*) (Bieluszewski et al., 2015). EAF1 copurifies with other NuA4 subunits, and physically interacts with YAF9A and YAF9B. Moreover, plants with decreased expression of *EAF1* show early flowering phenotype and low levels of *FLC*. These plants also present reduced H4K5Ac levels in the chromatin of *FT* and *FLC*, especially around the 5’ end. These results suggest that EAF1 is a member of the Arabidopsis NuA4 complex and it is required for the regulation of H4 acetylation levels in the chromatin of relevant flowering genes.

The observations described above show that histone acetylation plays a key role in the regulation of master genes of flowering. Also, there seems to be growing evidence that supports the existence of a NuA4 complex in Arabidopsis, and that the characterization of its putative subunits indicates that it is involved in the chromatin-mediated regulation of flowering time. For these reasons, we decided to investigate in more detail the role of the Arabidopsis NuA4-C by characterizing some representative components.

OBJECTIVES

OBJECTIVES

Our group is interested in understanding how chromatin remodeling mechanisms regulate development and particularly flowering time in the model plant *Arabidopsis thaliana*. The isolation and characterization of mutants in the components of SWR1 has shown that this complex represses flowering by participating in the chromatin-mediated activation of the floral repressor *FLC*. Studies in yeast have shown that SWR1 cooperates with NuA4 to activate the transcription of target genes, and recent studies support the existence of a NuA4 complex in Arabidopsis that might also be involved in the regulation of the floral transition. For these reasons, we decided to gain a deeper insight in the role exerted by different components of the putative Arabidopsis NuA4 complex in the control of flowering time, and proposed the following specific objectives:

1. Characterize the role of the Arabidopsis homologues of Tra1, Epl1 and Eaf6 in the regulation of flowering time.
2. Characterize the role of MRG1 and MRG2, the homologues of Eaf3, in the control of the floral transition, investigating their possible relationship with other chromatin regulators that modulate H3K36me3 and H3/H4 acetylation.
3. Analyze the effect of loss-of-function mutations in *MRG1* and *MRG2* on the Arabidopsis transcriptome.
4. Characterize the role of ING1 and ING2, the Arabidopsis homologues of Yng2, in the regulation of flowering time.
5. Analyze the existence of genetic relationships of *ING1* and *ING2* with genes encoding key regulators of flowering time, including some chromatin remodeling factors, and study the physical interactions of ING1 and ING2 with putative components of the NuA4 complex.
6. Gain a deeper understanding of the chromatin-mediated regulation of flowering target genes by ING1 and ING2.
7. Evaluate the misregulation of global gene expression caused by loss-of-function mutations in *ING1*, *ING2*, or both in Arabidopsis.

MATERIALS AND METHODS

Materials and methods

1. Plant material

In this work, we have used the *Arabidopsis thaliana* (L.) Heynh wild type (wt) accession Columbia (Col). We have also used different mutant, introgression and transgenic lines that are shown in Table 1. All mutant lines used in this study are in a Col genetic background and were obtained from the Arabidopsis Biological Resource Center (ABRC) (Columbus, OH, USA), from the Nottingham Arabidopsis Stock Center (NASC, UK), and from personal donations from other researchers.

Name	Background	Flowering phenotype	Reference
arp6-1 FRI	Col	Similar to wt	Dr. Crevillén, unpublished
atx1-2	Col	Early flowering	Pien et al, 2008
atxr7-2	Col	Early flowering	Tamada et al, 2009
axe1-5	Col	Late flowering	Murfett et al, 2001
Col *FRI SF2*	Col	Natural late flowering allele introgressed in Col	Lee and Amasino, 1995
eaf6-1	Col	n.a.	This work
eaf6-2	Col	Late flowering in SD	This work
ebs	Col	Early flowering	López-González et al, 2014
epl1-1	Col	Late flowering	This work
epl2-2	Col	Late flowering	This work
esd1-10	Col	Early flowering	Martín-Trillo et al, 2006
flc-3	Col	Early flowering	Michaels and Amasino, 2001
ft-10	Col	Late flowering	Yoo et al, 2005
ing1-1	Col	Early flowering in LD	This work
ing2-1	Col	Late flowering	This work
ing2-2	Col	Late flowering	This work
mrg1-2	Col	Similar to wt	This work
mrg2-4	Col	Similar to wt	This work
pHTA11::HTA11-GFP	Col	Similar to wt	Kumar and Wigge, 2010
pMRG2::MRG2-YFP	Col	Similar to wt	Bu et al, 2014
sdg8-1	Col	Early flowering	Zhao et al, 2005
swc6-1	Col	Early flowering	Lázaro et al, 2008
tra1-1	Col	Late flowering	This work
tra1-2	Col	Late flowering	This work
tra1-3	Col	Late flowering	This work
tra1-4	Col	Late flowering	This work
tra2-2	Col	Similar to wt	This work
tra2-4	Col	Similar to wt	This work

Table 1. Mutant, introgression and transgenic lines used in this work

2. Plant culture conditions

Arabidopsis seeds were sown in universal substrate and vermiculite (3:1 proportion), or plated on Petri dishes with MS or GM (MS medium supplemented with 2% sucrose) culture medium supplemented with 0.7% plant-agar (Murashige and Skoog, 1962) for *in vitro* culture.

Seeds sown in universal substrate were previously sterilized in a solution containing 70% commercial bleach, 0.1% Triton X-100, and washed with sterile water up to three times (Feinberg and Vogelstein, 1983; Shi et al., 2013). Alternatively, seeds for *in vitro* culture were sterilized by exposure to a solution containing 3% (v/v) HCl in commercial bleach, in a hermetically sealed container for 4h (Clough and Bent, 1998).

After sterilization and sowing, seeds were stratified at 4ºC for 3-4 days in darkness. For cultivation in universal substrate, we used constant controlled conditions of 22ºC and 65% relative humidity in growth chambers. Plants were illuminated with cold white fluorescent light (~120 µE m^{-2} s^{-1}). The photoperiodic conditions used were long days (LD: 16h light/8h darkness) and short days (SD: 8h light/16h darkness).

Seeds grown *in vitro* were grown in chambers with the following conditions: 22ºC, 65% relative humidity, and cold white fluorescent light.

3. Phenotypic analysis

3.1. Quantification of flowering time

Flowering time is directly related to the number of predetermined leaves at the moment of bolting (Koornneef et al., 1991). Based on this premise, we quantified the total leaf number at flowering except the cotyledons, that is, the sum of rosette leaves and cauline leaves of the main inflorescence in each plant. Data are presented as the mean value ± standard deviation. Sample size was at least 20 plants in SD and 30-50 plants in LD. All flowering time experiments were repeated at least three times.

3.2. Analysis of morphological traits

To analyze silique size, rosette size, leaf and flower morphology and silique sections, seeds were grown in LD and/or SD conditions, and the parameters of interest measured. Sample size was 50 siliques for determining silique size, 10 plants for rosette size and leaf morphology, and 50 flowers from 4 different plants for flower morphology. Silique sections were performed on 10 siliques from at least two different plants of each genotype. For quantitative traits, data are presented as the mean value ± standard deviation.

3.3. Chlorophyll extraction and quantification

Seeds sown on GM medium were grown *in vitro* for 10 days. 100 mg of fresh tissue were collected in Eppendorf tubes containing 1 mm-glass beads, and homogenized using a

SILAMAT vibrating mill (Schaan, Principality of Liechtenstein) for 5 s. 1 ml of 80% acetone was added to each sample and incubated overnight at 4ºC.

Absorbance at 646.6 nm and 663.6 nm was measured and total chlorophyll concentration per ml was calculated using the following formula (Moran and Porath, 1980), and represented as the mean value ± standard deviation:

$$\textbf{Total Chl (µg/ml)} = 17.76\ (A_{646.6}) + 7.34\ (A_{663.6})$$

4. Extraction and analysis of genomic DNA

To genotype Arabidopsis plants, genomic DNA was obtained from a leaf. The tissue was collected in Eppendorf tubes containing 1 mm-glass beads, frozen in liquid nitrogen and homogenized using a Ventura Mix 2 vibrating mill for 5 s. Genomic DNA was extracted following the protocol described by Bernatzky and Tanksley (1986). The isolated genomic DNA was used as a template in PCR reactions, using the specific molecular markers for each mutant allele shown in Table 2. In the genotyping of T-DNA lines, we verified the presence of the insertion by PCR, using the primers LBb1.3 (ATTTTGCCGATTTCGGAAC) for SALK lines, LB1 (GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC) for SAIL lines, and GABI8760 (GGGCTACACTGAATTGGTAGCTC) for GABI-KAT lines, combined with a primer flanking the insertion (Table 2).

Allele	Type	Forward	Reverse
atx1-2	T-DNA	CATCTCTTTTGTGGACTTGCTG	AACAATTTGTTCCTGCAATGTG
atxr7-2	T-DNA	CATCTCTTCAGGGTTCCTTCC	GGTTTCCTCAGATTCCTCAGC
axe1-5	Point mutation	AGAAGCGTTTTACACCACTGATA	TTAAGACGATGGAGGATTCACG
Col *FRI Sf2*	INDEL	AGATTTGCTGGATTTGATAAGG	ATATTTGATGTGCTCTCC
eaf6-1	T-DNA	CAGCGGAGATGCTCCTATTC	CTTGGAGGAAGACAAAAACCC
eaf6-2	T-DNA	GGGTTTTTGTCTTCCTCCAAG	TGCTTCTCTTTGCCTCTCTTG
epl1-1	T-DNA	GCCATGAAATCCCTCTTTTTC	TACTCCTACCCTTGGTTTGCC
epl2-2	T-DNA	AGCGTAAAAGATGGCAGAAGC	TACCAGTCTGTGAAGCCATCC
esd1-10	T-DNA	TGCATGGACTCTCAACCCTAC	CAGCCTGCAGATTTAGGTTTG
flc-3	INDEL	TAGAAAGAAATAAAGCGAGAAAAGGA	CCCAGGTAAGGAAAAGGCGTA
ft-10	T-DNA	CAGGTTCAAAACAAGCCAAGA	TAAGCTCAATGATATTCCCGTACA
ing1-1	T-DNA	CGCGATTTGGATAAAAGTTTG	AAGTCATCCCAGTTGATGCTG
ing2-1	T-DNA	TTTCAGCCAGTTTTGGTGTTG	ACCATTTCCCTTTGAATCTGG
ing2-2	T-DNA	TTGCCGTAAGGATACATGCTC	TAATCTCAATGTTTGTGGGGC
mrg1-2	T-DNA	TTGATTCTTGGTATTGTCGCC	TGGGTTAGTGCGGATAGATTG
mrg2-4	T-DNA	CTTCTTCAAGATGCCATCCAC	AAATGATTTCTTTTGGCAGGG
sdg8-1	T-DNA	CCTTCATCGCAATCGTAAATC	TTTTGCGCTAAACTAGTTGGG
swc6-1	T-DNA	AGGACAGGAACATGGAGATTG	AAGTTGTTAAAGGCCCAATGG
tra1-1	T-DNA	TTGAAGATGGTCTTCATTGCC	GCCAACGGATACCTATCAGTG
tra1-2	T-DNA	ATTTGTCCCTTTCTCAGGTGC	TGATTTGATCCTTTCCGTCAC
tra1-3	T-DNA	AAAGAACACGAGCCATGTGAC	GTGCCTCTACTGCAGCAATTC
tra1-4	T-DNA	CATCACTACCGAATCGAGAGC	AAGCTCTCCTTTCGTTTCCAG
tra2-2	T-DNA	AAACACAGTGAGACGGAATGG	TGAGATCATCCTCAACCATCC
tra2-4	T-DNA	CTTTGTTCAAACGATTCTGGG	AGGAACATAACAACAAGGGGG

Table 2. Molecular markers for the detection of the mutant alleles used in this work

The double and triple mutants analyzed in this work were obtained by genetic crosses between the parental single mutant lines and subsequent selection in the F_2 generation using molecular markers for each mutation. The selection of double and triple mutants was further confirmed by checking that there was no segregation of the parental lines in the F_3 generation.

5. GA sensitivity assays

The analysis of the sensitivity of *eaf6-2* mutants to gibberellin treatments was done with plants grown on universal substrate. Seven days after germination, the seedlings were sprayed with a solution of 100 µM GA_{4+7} (Duchefa), a bioactive form of GA. Control plants were sprayed with water (mock). Plants were sprayed every 7 days until they flowered, and their flowering time was quantified.

6. Protein structure modeling

The predicted protein structure of MRG1 and MRG2 was obtained by depositing their protein sequences into the SWISS-PROT/TrEMBL tool (http://swissmodel.expasy.org/), described by Schwede et al. (2003). The resulting structural models were visualized with Swiss-Pdb Viewer v4.01 (Guex and Peitsch, 1997).

7. Generation of plasmid constructs and transformation into bacterial strains

7.1. Generation of ING1 *and* ING2 *overexpression constructs*

A cDNA clone containing the full length CDS of *ING1* was obtained from the ABRC DNA stock center. The AttB1 and AttB2 sequences were attached to the CDS of *ING1* to adapt it for GateWay cloning (Invitrogen). To this end, we amplified *ING1* by PCR from this clone using the ING1 GW F and ING1 GW R primers (Table 3), and the resulting product was used as a template for a second PCR reaction with the AttB1 and AttB2 primers. Then, the PCR product was purified and the *ING1* CDS was introduced into the Gateway-compatible entry vector pDONR207 by a recombination reaction with BP clonase I (Invitrogen). The resulting clone was sequenced to verify that it did not contain any mutations. The *ING2* CDS was obtained from a cDNA clone from the ABRC DNA stock center in a Gateway-compatible entry vector and sequenced.

Primer	Sequence
ING1 GW F	GGAGATAGAACCATGTCATTCGCCGAGGAA
ING1 GW R	CAAGAAAGCTGGGTCTCATCGACCTTTCCTGCT
AttB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACC
AttB2	GGGGACCACTTTGTACAAGAAAGCTGGGTC

Table 3. Primers used for the cloning of ING1 in pDONR207

The *ING1* CDS was mobilized to the destination vector pEarleyGate 202, which harbors a 35S promoter and a FLAG epitope in N-terminal (Earley et al., 2006), by a recombination reaction with LR clonase I (Invitrogen). The *ING2* CDS was mobilized the destination vector pGWB18 (Nakagawa et al., 2007), which bears a 35S promoter and a 4xMYC epitope in N-terminal, also by an LR recombination reaction. Both resulting clones were checked with restriction enzymes and transformed into competent *Agrobacterium tumefaciens* cells (strain AGL0).

The constructs generated in this work were transformed and amplified in *Escherichia coli* cells (strain DH5α). These cells were grown in LB medium supplemented with the corresponding antibiotic (Sambrook et al., 1989) at 37ºC for 14-16h. *A. tumefaciens* cells were cultured at 28ºC for 48h. For the preparation of *E. coli* competent cells we used the "Z-competent *E. coli* transformation buffer set" kit (Zymo Research) and transformation was performed following the manufacturer's instructions. *A. tumefaciens* electrocompetent cells were prepared as described by Mersereau et al. (1990) and transformations by electroporation were done as described by Hofgen and Willmitzer (1988), using a Gene Pulser device (Biorad). Plasmid DNA was obtained using the standard protocol (Sambrook et al., 1989).

For the purification of DNA fragments for cloning approaches, these were separated by agarose-TBE electrophoresis and the bands were sliced out of the gel. The DNA in the gel slice was purified using the "FavorPrep GEL/PCR Purification Mini Kit" (Favorgen) and sequenced by the Secugen Sequencing service. The sequences obtained were analyzed with Chromas 1.45 and the BLAST tool from the Arabidopsis Information Resource website (www.arabidopsis.org).

7.2. Generation of ING1*,* ING2*,* EPL1*,* EPL2*,* HAM1 *and* EAF6 *constructs for yeast two-hybrid analysis*

The CDS of *EPL1*, *EPL2*, and *EAF6* were obtained from the ABRC DNA stock center in Gateway-compatible entry vectors. The CDS of *HAM1* had been cloned during the course of the TRANSPLANTA project, which aims to functionally characterize a collection of *A. thaliana* transcription factors (Coego et al., 2014), and was available in the laboratory.

The pGBT8 and pGAD vectors were used to generate fusions with the GAL4 Binding Domain (GBD) or GAL4 Activation Domain (GAD), respectively. *ING1*, *ING2* and *HAM1* were cloned in the pGBT8 vector, and *EPL1*, *EPL2* and *EAF6* were cloned in the pGAD vector with a LR recombination reaction, using the Gateway technology.

8. Generation of *Arabidopsis thaliana* transgenic plants

Arabidopsis thaliana transgenic plants were generated using the floral dip method described by Clough and Bent (1998), and the *Agrobacterium tumefaciens* strain AGL0. T0 plants were grown in LD conditions as previously described, until they developed the main inflorescence. These T0 plants were submerged for 5 minutes in infiltration medium containing a suspension of *A. tumefaciens* cells bearing the transgene construct of interest. The infiltrated

plants were then moved to growth chambers so they could develop T1 seeds. These T1 seeds were sterilized and sown at a density of 50 seeds/cm^2 in GM medium supplemented with the corresponding antibiotic or herbicide (kanamycin 50 µg/ml; hygromycin 40 µg/ml; phosphinothricin 10 µg/ml), and the resistant plants were transplanted to universal substrate. After selection of T2 seeds, 200 seeds of each T2 line were plated in the same conditions. Lines exhibiting a survival ratio in selective medium of 3:1 (resistant to sensitive) were selected as single-T-DNA-insertion lines and transplanted to universal substrate Finally, the T3 lines showing 100% of survival in selective medium were selected as homozygous.

9. Expression analyses

9.1. RNA extraction

Seeds were sterilized as previously described and sown in Petri dishes containing GM medium supplemented with 0.7% plant-agar at a density of 25 seeds/cm^2. Then, seeds were stratified and grown as described.

The seedling material was collected in Eppendorf tubes containing 1 mm-glass beads, frozen in liquid nitrogen and homogenized using a SILAMAT vibrating mill for 5 s. To analyze the expression of flowering genes, seedlings were collected at the last hour of the light period, ZT16 in LD and ZT8 in SD. RNA was isolated using the EZNA Plant RNA Kit (Omega Bio-tek) and following the manufacturer's instructions. 4 µg of RNA were treated with RNAse-free DNAse to eliminate traces of contaminant genomic DNA. Total RNA concentration was determined by spectrophotometry (Sambrook et al., 1989) using a NanoDrop ND-1000 (Thermo Scientific).

The integrity of the isolated RNA was checked electrophoretically, by loading 2 µg of RNA in a 1% agarose gel, containing formaldehyde/formamide (Sambrook et al., 1989). The buffer used as electrolyte was 1X MOPS (Sambrook et al., 1989). Samples were diluted in a loading buffer containing 1X MOPS, formaldehyde, formamide and ethidium bromide (Sambrook et al., 1989), and incubated at 65ºC for 5 minutes, before loading them in the agarose gel.

9.2. Analysis of mRNA expression

Total cDNA was synthesized using the "Superscript First-Strand synthesis for RT–PCR" kit (Invitrogen) following the manufacturer's instructions, using between 1 and 3 µg of total RNA as template and $Oligo(dT)_{20}$. The resulting cDNA was diluted 1:10 and 2 µl of diluted cDNA were used for each qPCR reaction, in a final volume of 12 µl.

The qPCR analyses were performed with the "Light Cycler FastStart DNA Master SYBR Green I" kit, following the protocol described by Roche. The primers used for the expression analyses are listed in Table 4. Relative expression levels were normalized to the expression of the housekeeping genes *β–ACTIN1* (*ACT1*) and *UBIQUITIN-CONJUGATING ENZYME 21* (*UBC21*) and represented as mean value ± standard deviation.

Gene	Forward	Reverse
ACT1	TGTCGCCATCCAAGCTGTTCTCT	GTGAGACACCATCACCAGAAT
EAF6	CGAAGAAAGGGAGAGGACAAT	GAGTGAACCATGGCCATACA
EPL1	GCCAGTTATGCTGTTCACGA	TGTGGGCTTGGTTTAGAGGA
EPL2	AGACGACAGTCACAGCATGG	GAAACCGACTTGGAGGTTGA
FLC	AGCCAAGAAGACCGAACTCA	TTTGTCCAGCAGGTGACATC
FT	CTGGAACAACCTTTGGCAAT	AGCCACTCTCCCTCTGACAA
FUL	TTGCAAGATCACAACAATTCGCTTCT	GAGAGTTTGGTTCCGTCAACGACGAT
FWA	CTCTGGTCAAGACTCTTATGG	ATTCTGCTTGAATCTGTTGG
ING1 DOWNSTREAM	AGTTAGCTTTGGCGAGATGG	TCATCGACCTTTCCTGCTCT
ING1 UPSTREAM	GAATTTGAAGCTAATCTTGTTTCG	TTCACAGCGTTGTTCATTTTG
ING2 DOWNSTREAM	AGCGGAAAAGCGTAAGTCCT	AAGGACACCTGATGGCAGAC
ING2 UPSTREAM	AGCTTCGTTCTCAATCCCAAT	TAGCTCTGCAGGGAAAGTGC
MAF1/FLM	TCACCTTAAACTCAAAGCCTGATTC	CAAACTCTGATCTTGTCTCCGAAG
MAF2	CATTGTGGGTCTCCGGTGATTAG	GATGAGACCATTGCGTCGTTTG
MAF4	GCTTCTCAAGTAACCACCATCAC	CTTGGATGACTTTTCCGTAGCAG
MAF5	CATGGATTGTGCTAGAAAACAACTG	GCTTCACTCTTCCGACACATCTAATC
MRG1	GAAAGAGAGGCGACAATACCA	TGGCAACTTGACAAAGAGACG
MRG2	AGGCTTGCGTTGCTACTTTG	GCAACTCCGGTAATTTCACAA
PHE1	GAACCGTAATTCTCAGATTCG	CAACCCTACGAATAACACCA
PI	GAACGCAAACAACAGAGTGG	TGGTCCAACATAGCACCAAG
SOC1	CGTTATCTGAGGCATACTAAGGATCG	CCTTAAACACTTGAGTCTTTCTTGC
TRA1	ATCACAGGGCAAATCTCACC	TTCCAGAATTTTTGGCGAAC
TRA2	CGTGATGAATTGTTGTCTTGG	GGCTCCACCGTATTCTCATC
UBC21	CTGCGACTCAGGGAATCTTCTAA	TTGTGCCATTGAATTGAACCC

Table 4. Primers used in qPCR assays for expression analyses

9.3. Global transcriptomic analyses

We analyzed which genes are misexpressed in *ing1-1*, *ing2-2*, *ing1-1 ing2-2* and *mrg1-2 mrg2-4* relative to Col performing global transcriptomic analyses by RNA sequencing (RNA-Seq). Heatmap diagrams were generated using MultiExperiment Viewer (MeV v4.9) (Saeed et al., 2006). Gene Ontology (GO) analysis among misregulated genes were performed using the GeneCoDis, a web-based tool for finding significant concurrent annotations in gene lists (Carmona-Saez et al., 2007), attending to biological process and molecular function categories.

RNA from 10-day-old seedlings grown in LD and harvested at ZT16 was extracted using the "EZNA Plant RNA Kit" (Omega Bio-tek). RNA was extracted from three independent replicates for each genotype and treated with "Turbo DNA-free kit" (Ambion). Library preparation, sequencing and bioinformatics analyses were performed by Beijing Genomics Institute (BGI).

10. Yeast two-hybrid analyses

The analyses of protein interactions by yeast two-hybrid (Y2H) were carried out on the yeast strain pJ694α, using the "MatchMaker Two-Hybrid System" (Clontech). The constructs of *ING1*, *ING2* and *HAM1* in pGBT8, and *EPL1*, *EPL2* and *EAF6* in pGAD were transformed into yeast cells. Cells transformed with these constructs were selected in medium lacking leucine and tryptophan (-LW), and protein interactions were assayed in selective medium lacking leucine, tryptophan and histidine (-LWH), alone or supplemented with different concentrations of the histidine synthesis inhibitor 3-amino-1.2.4-triazole (3AT).

11. Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation assays were performed as described by Song et al. (2014). Plant material was grown on GM plates for 10 days in LD and harvested at ZT16. The protein-DNA complexes were crosslinked with formaldehyde and vacuum for 20-30 min. Chromatin was extracted and fragmented with a Bioruptor Plus sonication device (Diagenode)(30 cycles: 30 s on, 30 s off) as described (Song et al, 2014), and later immunoprecipitated with antibody-coated Dynabeads Protein A (for mouse antibodies) or Dynabeads Protein G (for rabbit antibodies) (Life technologies). No-antibody-coated beads were used as negative controls. The antibodies used are listed in Table 5. Samples were washed as described by Song et al (2014) and crosslinking was reverted with a 10% Chelex resin. DNA was purified with QIAquick kit columns (QIAGEN).

Epitope	Manufacturer	Host species	Amount per sample
H4K5,8,12,16 Ac	Millipore	Rabbit (polyclonal)	4 µg
H3K9,14 Ac	Millipore	Rabbit (polyclonal)	4 µg
GFP	Life technologies	Rabbit (polyclonal)	5 µg
H3K4me3	Millipore	Mouse (monoclonal)	4 µg
H3K27me3	Millipore	Rabbit (polyclonal)	4 µg
FLAG (Clone M2)	Sigma	Mouse (monoclonal)	10 µg
MYC (Clone 4A6)	Millipore	Mouse (monoclonal)	10 µg

Table 5. Antibodies used for ChIP experiments. The epitope recognized by the antibody, the manufacturer, the host species and the amount used per sample are indicated.

The GFP ChIP experiments shown in Fig. 27 and 58 were performed as described in Yamaguchi et al. (2014).

The abundance of immunoprecipitated DNA was measured by qPCR, using the primers listed in Table 6. Results were represented as input % or fold enrichment. Fold enrichment was calculated by dividing the values of input % of each region by the values of input % of a control gene (*ACT2* or the retrotransposon *Ta3*). Data are presented as the mean value ± standard deviation.

Name	Locus	Forward	Reverse
ACT2	At3g18780	TGTCGCCATCCAAGCTGTTCTCT	GTGAGACACCATCACCAGAAT
At4g07700	At4g07700	GCGAAGTTGCTGTGAACAAA	TACATTGAGTTTGGCCGATG
FLC1	At5g10140	ACTATGTAGGCACGACTTTGGTAAC	TGCAGAAAGAACCTCCACTCTAC
FLC2	At5g10140	GCCCGACGAAGAAAAAGTAG	TCCTCAGGTTTGGGTTCAAG
FLC3	At5g10140	CGACAAGTCACCTTCTCCAAA	AGGGGGAACAAATGAAAACC
FLC4	At5g10140	GGCGGATCTCTTGTTGTTTC	CTTCTTCACGACATTGTTCTTCC
FLC5	At5g10140	TCATTGGATCTCTCGGATTTG	AGGTCCACAGCAAAGATAGGAA
FLC6	At5g10140	TTGACAATCCACAACCTCAATC	TCAATTTCCTAGAGGCACCAA
FLC7	At5g10140	GGGGCTGCGTTTACATTTTA	GTGATAGCGCTGGCTTTGAT
FLC10	At5g10140	TGGTTGTTATTTGGTGGTGTG	ATCTCCATCTCAGCTTCTGCTC
FT3	At1g65480	GTGGCTACCAAGTGGGAGAT	TCACACATTGTCGTCTTATTTCA
FT4	At1g65480	TGATTTCACCGACCCGAGTT	AGGCATGAACCCTCTACACATATTTATA
FT5	At1g65480	GAGACCCTCTTATAGTAAGCAGAGTT	CCTGAGGTCTTCTCCACCA
FT6	At1g65480	GTTCTTTCACTTGAACTCCCTTTTG	CCCAAGAAATATTTTCAGTATACCCC
FT7	At1g65480	TTCTTTTTTCATGAAGATGGACCC	TAATCACTTATGCAAGAAGTTGGTGG
Ta3	*Ta3*	TAGGGTTCTTAGTTGATCTTGTATTGAGCTC	TTTGCTCTCAAACTCTCAATTGAAGTTT

Table 6. Primers used in ChIP experiments.

12. Protein extraction and western blot analysis

To analyze the transgene protein expression and abundance in the transgenic lines generated, seeds were sterilized, plated on GM medium and grown in vitro for 10 days as described above. The tissue was collected in Eppendorf tubes containing 1mm-glass beads frozen in liquid nitrogen and homogenized using a SILAMAT vibrating mill for 5 s. Samples were resuspended in protein extraction buffer containing 10% glycerol, 150 mM NaCl, 50 mM Tris-HCl pH7.0, 0.1% Nonidet P40, 0.5% CHAPS (Duchefa), and a protease inhibitor cocktail (Roche), sonicated for 10 s with a UP100H sonicator (Hielscher) three times, incubated for 30 min at 4ºC and centrifuged at 13200 rpm at 4ºC. Protein concentration in each sample was measured using a "Quick Start Bradford 1X Dye Reagent" (Bio-Rad). 20 µg of the protein extracts were separated by SDS-PAGE in 12% polyacrylamide gels and then transferred to Immobilon membranes (Millipore), following the manufacturer's instructions. The membranes were blocked with 3% BSA overnight at 4ºC and the proteins were detected using an α-MYC antibody (Millipore), an anti-mouse-HRP secondary antibody (Santa Cruz) and an "Immobilon Western" kit (Millipore), following the manufacturer's instructions

RESULTS

RESULTS

1. Characterization of Arabidopsis homologues of the yeast NuA4 complex subunits Tra1, Epl1 and EAF6

1.1. Characterization of Tra1 homologues in Arabidopsis

Tra1 is the *Saccharomyces cerevisiae* homolog of the human protein TRRAP (TRansformation/tRanscription domain-Associated Protein). TRRAP was originally identified as an interactor of the c-Myc and E2F transcription factors (McMahon et al., 1998) and was later found as part of the SAGA histone acetyltransferase complex (Grant et al., 1998). *S. cerevisiae* Tra1 is a 400-kDa protein found in three different coactivator complexes: SAGA, SLIK and NuA4, being essential for viability, as its complete knock-out leads to lethality (Saleh et al., 1998). Tra1 is a relatively large protein that accounts for approximately half of the total mass of the yeast NuA4 complex (Chittuluru et al., 2011). This protein belongs to the phosphoinositide 3 kinase-related family and it bears three domains: a FAT (FRAP, ATM and TRRAP) domain, a PI3K domain that lacks some of the essential residues for its enzymatic activity, and a FATC domain (FAT C-terminal), involved in the maintenance of the three-dimensional structure of the protein (Knutson and Hahn, 2011; Hoke et al., 2010). The lack of kinase activity suggests that Tra1 might have structural functions, acting as a scaffold for protein complex assembly. However, Tra1 and TRRAP also play regulatory roles by interacting with transcription factors and recruiting co-activator complexes to specific loci (Brown et al., 2001; Murr et al., 2007). Yeast Tra1 has been shown to interact with transcriptional activators such as Gcn4, Hap4 and Gal4 (Brown et al., 2001), whereas human TRRAP can interact with important transcriptional regulators like c-Myc, E2F and p53 (McMahon et al., 1998; Lang et al., 2001). Given the regulatory role of Tra1/TRRAP in yeast and humans, it is possible that their counterparts in Arabidopsis might also have regulatory functions in the context of a putative NuA4 complex. To investigate this, we aimed to identify and characterize the putative Arabidopsis orthologues of Tra1.

The Arabidopsis genome contains two genes that share homology with *Tra1*/*TRRAP* that we have designated as *AtTRA1* and *AtTRA2*. *AtTRA1* (At4g36080) is a large gene with 34 exons (Fig. 5A). To functionally characterize *AtTRA1* and unveil its possible role in the regulation of flowering time, we took a reverse genetics approach and searched public databases for T-DNA insertion lines that might result in loss-of-function alleles of *AtTRA1*. As a result, we identified four insertion lines, namely SALK_105303, SALK_087867, SALK_053301, and SAIL_805_H09, referred to as *tra1-1*, *tra1-2*, *tra1-3* and *tra1-4*, respectively. The T-DNA insertions of *tra1-1* and *tra1-2* are located in exon 24 and exon 7, respectively, whereas the *tra1-3* and *tra1-4* alleles both carry a T-DNA insertion in exon 26 (Fig. 5A). To determine if *tra1-1* was a null allele, we analyzed the expression of *TRA1* in the *tra1-1* mutant line by qPCR. We could barely detect any expression of *TRA1* in the mutant line compared to the wt Col (Fig. 5B), confirming that *tra1-1* is likely a null allele of *AtTRA1*.

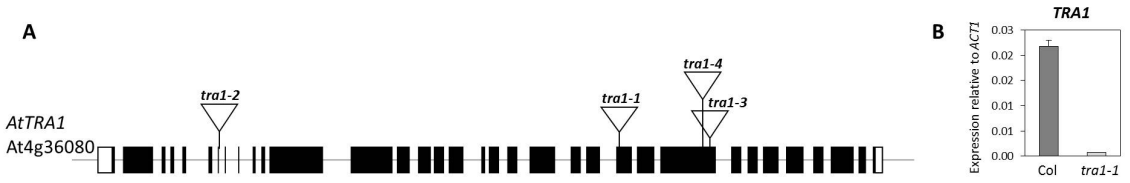

Figure 5. The *TRA1* gene in *Arabidopsis thaliana*. (A) Gene structure of *AtTRA*1 and T-DNA insertion lines. Boxes represent exons and the black line represents the introns. The white boxes represent the 5' and 3' UTRs (left and right, respectively). The inverted triangles represent the position of the T-DNA insertions in the *tra1-1*, *tra1-2*, *tra1-3* and *tra1-4* alleles. **(B)** Expression of *TRA1* in the mutant line *tra1-1* analyzed by qPCR in 10-day-old seedlings grown in LD conditions. *ACT1* expression was used as a control.

1.1.1. Loss-of-function mutants of AtTRA1 display a late flowering phenotype in LD and SD.

To investigate the possible role of *AtTRA1* in the regulation of the floral transition in Arabidopsis, we analyzed the flowering phenotype of the four previously isolated mutant lines under inductive LD photoperiods. We observed a late flowering phenotype in all four alleles, which was more noticeable in the case of *tra1-1* and *tra1-4* (Fig. 6A, B). We also analyzed the phenotype of *tra1-1* plants grown under non-inductive SD photoperiods, and we observed that, as in LD, *tra1-1* plants also display a late flowering phenotype under these conditions (Fig. 6C, D). The late flowering phenotype observed for loss-of-function alleles of *AtTRA1* indicates that the protein encoded by this gene plays a role in the induction of flowering in Arabidopsis, independently of the photoperiod.

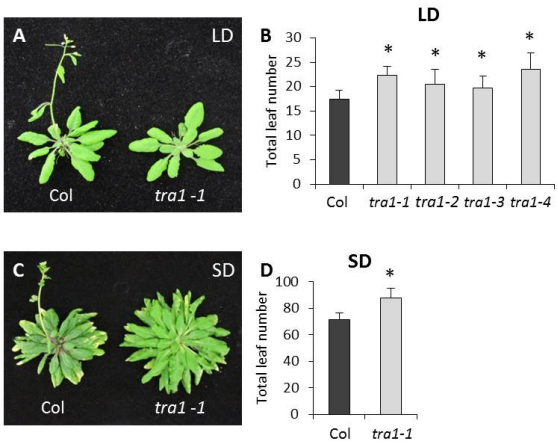

Figure 6. Mutations in *AtTRA1* cause a late flowering phenotype under LD and SD. (A) Flowering time phenotype of *tra1-1* plants under LD. **(B)** Total leaf number at flowering of Col, *tra1-1*, *tra1-2*, *tra1-3* and *tra1-4* plants grown in LD conditions. **(C)** Flowering time phenotype of *tra1-1* plants under SD. **(D)** Quantification of total leaf number of Col and *tra1-1* plants grown in SD conditions. * $P < 0.05$ with Student's t-test.

1.1.2. tra1-1 *mutant plants show altered expression of flowering genes.*

Given that the *tra1-1* mutant plants show a delay in the floral transition, we analyzed the expression of key regulators of flowering such as the floral repressor *FLC* and the floral integrators *FT* and *SOC1* in LD conditions. We observed that the late flowering phenotype displayed by the *tra1-1* mutant correlates with a slight increase in the expression of *FLC* and a decrease in the expression of *FT* and *SOC1* (Fig. 7). This result indicates that TRA1 activates the floral transition by participating in the regulation of the master regulators of flowering *FLC*, *FT* and *SOC1*.

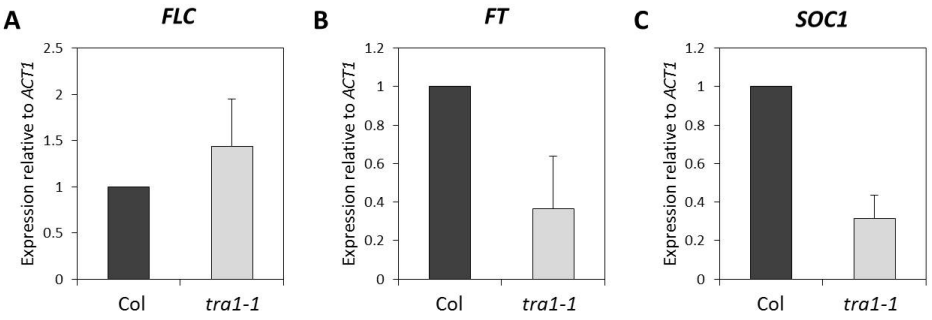

Figure 7. *TRA1* is required for the regulation of *FLC*, *FT* and *SOC1* under LD. Expression of *FLC* **(A)**, *FT* **(B)** and *SOC1* **(C)** in Col and *tra1-1*, analyzed by qPCR in seedlings grown in LD conditions for 9 days. *ACT1* expression was used as a control. Values in Col were set to 1.

1.1.3. Mutations in AtTRA2 *do not cause alterations in flowering time.*

AtTRA2 (At2g17930) is also a large gene containing 35 exons (Fig. 8A). To determine if *AtTRA2* plays a role in the regulation of flowering, we identified and isolated four T-DNA insertion lines for this locus, and two of them were further analyzed: SALK_069349 and SALK_114151, that we designated *tra2-2* and *tra2-4*, respectively. In the *tra2-2* allele, the insertion is located in intron 33, whereas in the *tra2-4* it is located in exon 2 (Fig. 8A). To test if these insertions could give rise to loss-of-function alleles of *TRA2*, we analyzed the expression of *TRA2* in these mutant lines. Both mutations abolish almost completely the expression of this gene, suggesting that *tra2-2* and *tra2-4* are likely null alleles of *TRA2* (Fig. 8B). We then analyzed the flowering phenotype of these mutants under LD photoperiods. In this case, we could not detect any significant difference in leaf number in these mutants as compared to the wt. This could be due to functional redundancy between *TRA1* and *TRA2*, and in the absence of TRA2, TRA1 might be performing its functions, resulting in the wt phenotype observed in *tra2* mutants. Further analyses involving the *tra1 tra2* double mutant will be necessary to reveal a possible functional redundancy between *TRA1* and *TRA2*.

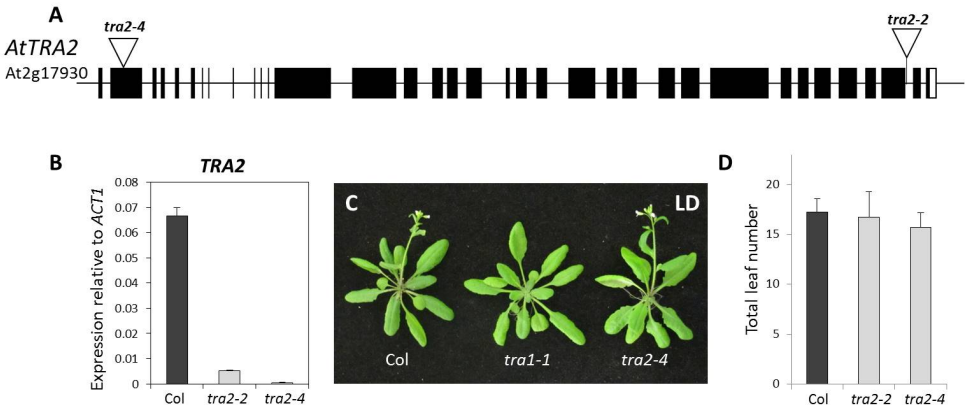

Figure 8. The Arabidopsis *TRA2* gene and flowering phenotype of *tra2* mutants. (A) Gene structure of *AtTRA2* and T-DNA insertion lines. Boxes represent exons and the black line represents the introns. The white box represents the 3' UTR. The inverted triangles represent the position of the T-DNA insertions in the *tra2-2* and *tra2-4* alleles. **(B)** Expression of *TRA2* in Col and the mutant lines *tra2-2* and *tra2-4*, analyzed by qPCR in 10-day-old seedlings grown in LD conditions. *ACT1* expression was used as a control. **(C)** Flowering time phenotype of Col, *tra1-1* and *tra2-4* plants in LD. **(D)** Total leaf number at flowering in Col, *tra2-2* and *tra2-4* plants grown in LD.

1.2. Characterization of Epl1 homologues in Arabidopsis

As mentioned above, *S. cerevisiae* NuA4 can exist in a large, 13-subunit complex, which accounts for targeted H2A and H4 acetylation, and in a smaller, 4-subunit Piccolo-NuA4, which is responsible for non-targeted acetylation. Yeast Piccolo-NuA4 is formed by Epl1, Eaf6, Yng2 and Esa1. The catalytic subunit Esa1 alone can only acetylate naked histones, but it is unable to acetylate nucleosomes (Boudreault et al., 2003), which indicates that other subunits are required for NuA4 to act on physiological chromatin substrates. Epl1 can independently bind Esa1 and Yng2, defining the structure of the Piccolo subcomplex and linking it to the rest of the complex (Chittuluru et al., 2011). Epl1 is also responsible for the strong preference of Piccolo for chromatin over free histones (Boudreault et al., 2003), therefore playing a fundamental role in NuA4 function. Unlike Tra1, Epl1 is not shared with other protein complexes and is only found in NuA4. Loss of function of Epl1 causes cells to accumulate in G2/M, leads to a reduction in global H2A and H4 acetylation and causes lethality as in the case of Esa1 mutants. This indicates that NuA4-mediated acetylation is essential for cell viability and highlights the pivotal role of Epl1 in NuA4 function (Boudreault et al., 2003).

1.2.1. *Mutations in* AtEPL1 *cause a late flowering phenotype in LD and SD.*

To investigate the possible role of Arabidopsis homologues of Epl1 in the regulation of flowering time we searched for genes with homology to *Epl1* in the Arabidopsis genome. We found two genes that we named *AtEPL1* and *AtEPL2*. *EPL1* is encoded by At1g16690 and has 12 exons (Fig. 9A). We identified a T-DNA insertion line from the SALK collection, SAIL_239_D12, carrying an insertion in exon 5, that we designated *epl1-1*. We analyzed the expression of *EPL1* in this mutant line and we observed that the *epl1-1* mutation abolishes almost completely the expression of this gene (Fig. 9B). Then, we examined the flowering time phenotype of this

mutant under LD and SD conditions. We observed that, as for *tra1* mutants, *epl1-1* plants show a delay in the floral transition both under LD (Fig. 9C, D) and SD (Fig. 9E), indicating that EPL1 also acts as an activator of flowering.

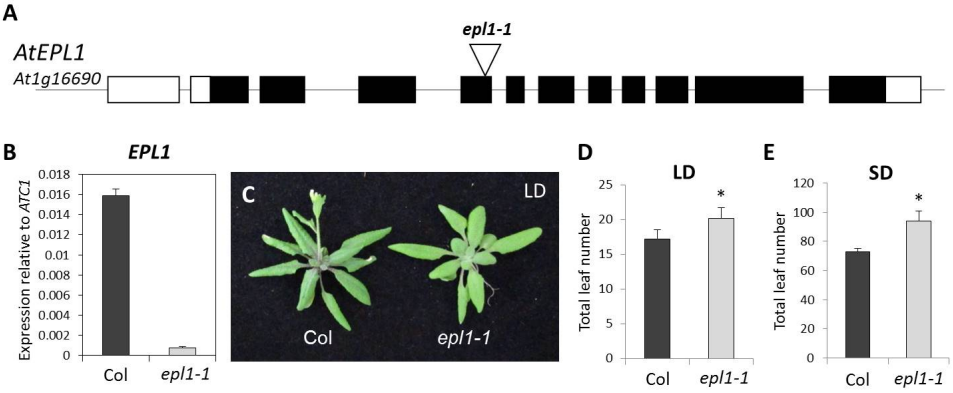

Figure 9. The Arabidopsis *EPL1* gene and flowering phenotype of the *epl1-1* mutant. (A) Gene structure of *AtEPL1* and T-DNA insertion line. Boxes represent exons and the black line represents introns. The white boxes represent the 5' and 3' UTRs (left and right, respectively). The inverted triangle represents the position of the T-DNA insertion in the *epl1-1* mutant. **(B)** Expression of *EPL1* in Col and *epl1-1*, analyzed by qPCR in 10-day-old seedlings grown in LD conditions. *ACT1* expression was used as a control. **(C)** Flowering time phenotype of Col and *epl1-1* plants in LD. **(D, E)** Total leaf number at flowering in Col and *epl1-1* plants grown in LD **(D)** and SD **(E)**. *P < 0.01 with Student's *t*-test.

1.2.2. Mutations in AtEPL2 *cause a late flowering phenotype in LD.*

EPL2 corresponds to At1g79020 and has 12 exons (Fig. 10A). Both *EPL1* and *EPL2* have the same number of exons and introns and a very similar gene structure (Fig. 9A and 10A). Their protein sequences are also highly similar, 75.85% according to the CLUSTALW tool from EMBL-EBI (not shown), which indicates a high degree of conservation between them and suggests they are close homologues. To investigate the function of *EPL2*, we identified an insertion line from the SALK collection (SALK_094941) that bears a T-DNA insertion in intron 9 of *AtEPL2* (Fig. 10A). Then, we checked the expression of *EPL2* in this mutant and found a reduction of approximately 70% in *epl2-2* plants. We also observed that the expression of *EPL2* is only slightly reduced in the *epl1-1* mutant, suggesting that EPL1 does not seem to regulate the expression of *EPL2* (Fig. 10B).

We analyzed the effect of the *epl2-2* mutation on flowering time and we could observe that, as in the case of *epl1-1*, *epl2-2* mutant plants show a late flowering phenotype under LD conditions (Fig. 10C), indicating that EPL2 also acts as an activator of flowering. The high degree of homology between *EPL1* and *EPL2* suggests that they might be partially redundant. To address this question we crossed *epl1-1* and *epl2-2* in order to generate an *epl1-1 epl2-2* double mutant. We genotyped 60 plants in the F2 population but could not retrieve any plant homozygous for both mutations. Given that mutations in yeast Epl1 and Drosophila E(Pc) result in lethality, we hypothesized that this could also be the case in Arabidopsis. To test this, we identified a plant homozygous for *epl1-1* and heterozygous for *epl2-2* and analyzed the seeds inside its siliques. We observed a number of aborted seeds in the siliques of the *epl1-1* $^{-/-}$

epl2-2 $^{+/-}$ plant that were not present in wt Col plants (Fig. 10D, E, F), which could correspond to double homozygous plants unable to complete their development due to embryo lethality.

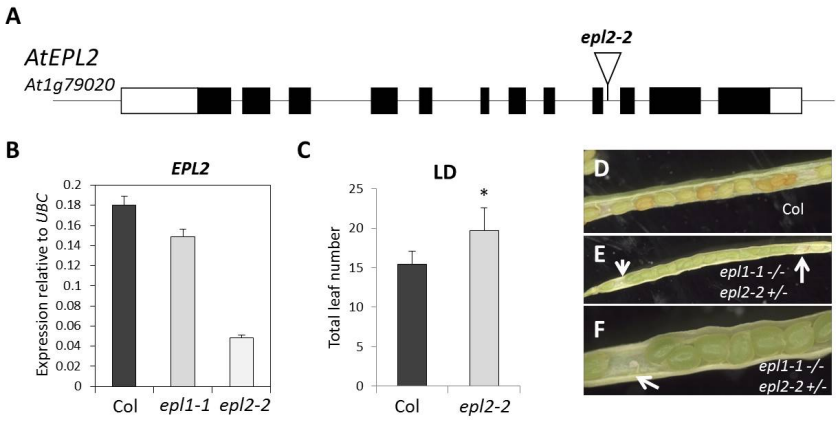

Figure 10. Arabidopsis *EPL2* regulates flowering time under LD. (A) Gene structure of *AtEPL2* and T-DNA insertion line. Boxes represent exons and the black line represents introns. The white boxes represent the 5' and 3' UTRs (left and right, respectively). The inverted triangle represents the position of the T-DNA insertion in the *epl2-2* mutant. **(B)** Expression of *EPL2* in Col, *epl1-1* and *epl2-2*, analyzed by qPCR in 10-day-old seedlings grown in LD conditions. *UBC* expression was used as a control. **(C)** Total leaf number at flowering in Col and *epl2-2* plants grown in LD. **(D, E, F) (D)** Longitudinal section of siliques from Col plants **(D)** and plants homozygous for *epl1-1* and heterozygous for *epl2-2* **(E, F)**. Aborted seeds can be observed in E and F (arrows). *P < 0.01 with Student's *t*-test.

1.2.3. Mutations in EPL1 *and* EPL2 *cause deregulation of* FLC *and* FT *expression*

To further understand the molecular basis of the activation of flowering mediated by EPL1 and EPL2 in Arabidopsis, we analyzed the expression of the floral repressor *FLC* and the floral integrator *FT* in *epl1-1* and *epl2-2* mutants under LD conditions (Fig. 11). We observed an upregulation in the expression of *FLC* in *epl1-1* and *epl2-2*, which was more pronounced in the case of *epl1-1*, and also a downregulation of *FT* expression in both mutant alleles, compared to wt Col plants. The upregulation of *FLC* and the downregulation of *FT* are consistent with the late flowering phenotype observed in these mutants and point to a regulation of EPL1 and EPL2 over these master regulators of flowering.

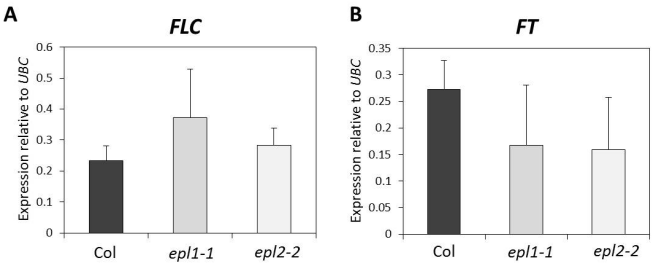

Figure 11. Mutations in *EPL1* and *EPL2* cause deregulation of *FLC* and *FT*. *FLC* **(A)** and *FT* **(B)** expression in Col, *epl1-1* and *epl2-2*, analyzed by qPCR in 10-day-old seedlings grown in LD conditions. Samples were harvested at ZT16. *UBC* expression was used as a control.

1.3. Characterization of Eaf6 homologues in Arabidopsis

As mentioned above, Eaf6 is a component of the Piccolo NuA4 subcomplex in *S. cerevisiae*, but it is also found in the NuA3 HAT complex that acetylates H3 (Taverna et al., 2006). This small 13-kDa protein with a leucine zipper region is present in all MYST-ING HAT complexes, but its function is currently unknown. Unlike all the putative Arabidopsis NuA4 homologue subunits characterized above, there is only one gene in the Arabidopsis genome that shares homology with yeast Eaf6. The Arabidopsis homologue of *Eaf6*, which we have named *AtEAF6*, is encoded by At4g14385. The fact that this gene is not duplicated makes it a good candidate to study the functions of the Piccolo NuA4 subcomplex in the model species Arabidopsis.

1.3.1. *Mutations in* EAF6 *cause developmental alterations.*

The *AtEAF6* gene contains 6 exons; to deepen in its putative functions, we identified and isolated two T-DNA insertion lines, namely SALK_075841 and SALK_026622, that we designated *eaf6-1* and *eaf6-2*, respectively (Fig. 12A). The insertion is located in the 5'-UTR in *eaf6-1* and in intron 3 in *eaf6-2*. We first checked the expression of *EAF6* in these mutant lines and observed that, while the *eaf6-1* mutation does not seem to have any effect in the expression of this gene, in the *eaf6-2* allele the expression of *EAF6* drops to almost undetectable levels, indicating that *eaf6-2* is likely a null allele of *AtEAF6* (Fig. 12B). For this reason, we decided to focus on the *eaf6-2* allele to further characterize the roles carried out by *EAF6* in Arabidopsis development.

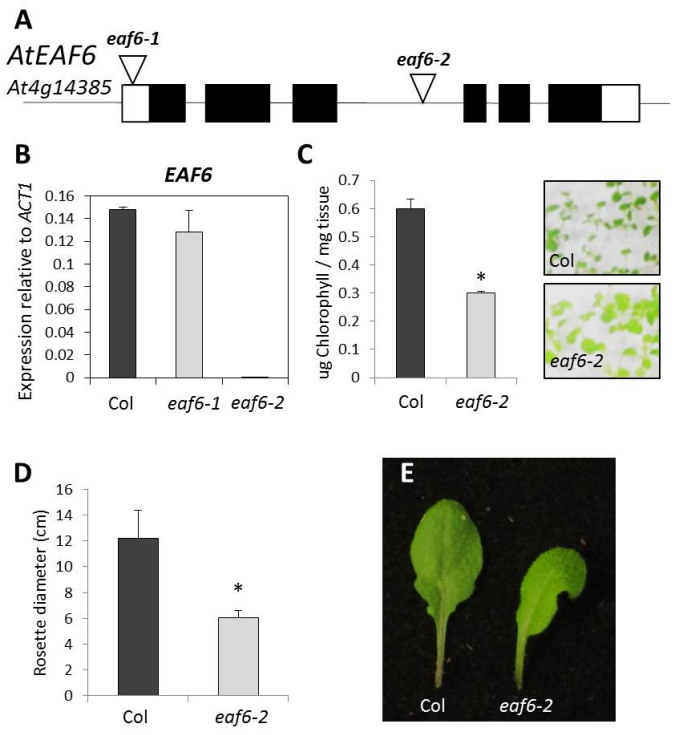

Figure 12. Mutations in *AtEAF6* cause developmental defects. (A) Gene structure of *AtEAF6* and T-DNA insertion lines. Boxes represent exons and the black line represents introns. The white boxes represent the 5' and 3' UTRs (left and right, respectively). The inverted triangles represent the position of the T-DNA insertions in the *eaf6-1* and *eaf6-2* alleles. **(B)** Expression of *EAF6* in Col, *eaf6-1* and *eaf6-2*, analyzed by qPCR in 10-day-old seedlings grown in LD conditions. *ACT1* expression was used as a control. **(C)** Quantification of the chlorophyll concentration in Col and *eaf6-2* in µg per mg of tissue, and images of Col and *eaf6-2* seedlings, which show symptoms of chlorosis. **(D)** Quantification of rosette diameter of Col and *eaf6-2* plants grown in SD. **(E)** Leaf phenotype of Col and *eaf-2* plants grown in LD. Sixth rosette leaves are shown. *P < 0.01 wit Student's *t*-test.

The phenotypic analysis showed that *eaf6-2* mutant seedlings display a paler color compared to wt, showing symptoms of chlorosis (Fig. 12C). To confirm that this chlorotic phenotype correlated with a reduction in the amount of chlorophylls, we quantified the concentration of these pigments in wt and *eaf6-2* seedlings. We observed that in the *eaf6-2* mutant, the concentration of chlorophylls was indeed reduced by approximately 50%, compared to Col plants (Fig. 12C).

We also noticed a reduced plant size in *eaf6-2* mutant plants when grown in SD conditions. The measurement of the rosette size of these plants revealed a reduction of approximately 50% in rosette diameter compared to Col plants (Fig. 12D and 13C). Furthermore, we also observed some alterations in leaf shape in the *eaf6-2* plants, which display an increased frequency of leaf curling (Fig. 12E).

1.3.2. eaf6-2 mutants show a late flowering phenotype in SD.

To test if mutations in *AtEAF6* lead to alterations in flowering time, we analyzed the flowering phenotype of the *eaf6-2* mutant grown under LD and SD conditions. We did not observe any difference in leaf number with wt in *eaf6-2* plants grown in LD (Fig. 13A, B). However, under SD conditions, *eaf6-2* plants produce a much higher number of leaves before bolting than Col plants (Fig. 13C, D). In fact, under these conditions, many of these plants are unable to complete their life cycle and die before flowering (not shown). The late flowering phenotype of *eaf6-2* indicates that, as observed for TRA1, EPL1, and EPL2, EAF6 is also involved in the activation of flowering in Arabidopsis, at least under SD.

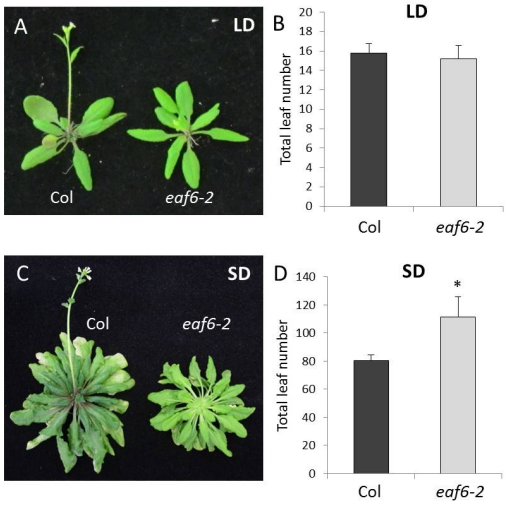

Figure 13. *eaf6-2* mutants show a late flowering phenotype in SD. (A) Flowering time phenotype of Col and *eaf6-2* plants grown in LD. **(B)** Total leaf number at flowering of Col and *eaf6-2* plants in LD. **(C)** Flowering time phenotype of Col and *eaf6-2* plants grown in SD. **(D)** Total leaf number at flowering of Col and *eaf6-2* plants in SD. *P < 0.05 with Student's *t* test.

1.3.3. The expression of flowering time genes is altered in eaf6-2 mutants.

To find out whether the alterations in flowering time observed in *eaf6-2* mutant plants were caused by misregulation of master floral regulators, we analyzed the expression of *FLC* and *FT* in Col and *eaf6-2* plants under LD and SD. We grew plants for 10 days under LD conditions and harvested the samples at ZT16. We detected no changes in the expression of these genes, which is consistent with the fact that in LD, the *eaf6-2* mutant flowered at the

same time as the wt (Fig. 14A, B). We also checked the expression of these genes under SD. For this purpose, we grew Col and *eaf6-2* plants for 20 and 25 days and collected the samples at ZT8. In these conditions, the *eaf6-2* mutant showed a strong upregulation of *FLC* at day 20, and a strong downregulation of *FT* at both day 20 and day 25 (Fig. 14C, D), which is in agreement with the late flowering phenotype observed in this mutant in SD.

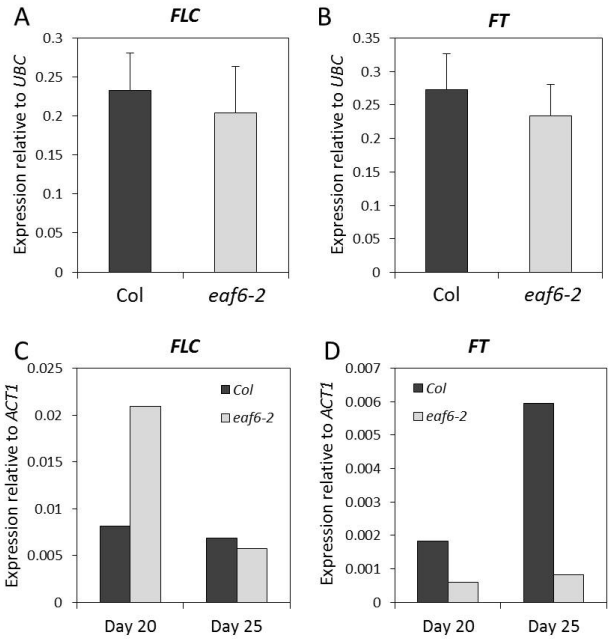

Figure 14. EAF6 is required for the regulation of *FLC* and *FT* under LD and SD conditions. *FLC* **(A)** and *FT* **(B)** expression in Col and *eaf6-2* plants grown in 10-day-old LD conditions. Samples were taken at ZT16. *UBC* expression was used as a control. *FLC* **(C)** and *FT* **(D)** expression in Col and *eaf6-2* plants grown in SD conditions for 20 and 25 days. Samples were taken at ZT8. *ACT1* expression was used as a control.

1.3.4. Exogenous GA can partially rescue the late flowering phenotype of eaf6-2 in SD.

Gibberellins play a central role in the induction of flowering in Arabidopsis, especially under non-inductive SD conditions. Mutants defective in the biosynthesis of this phytohormone, such as *ga1-3*, show a slight delay in flowering in LD and are unable to flower in SD (Wilson et al., 1992; Reeves and Coupland, 2001). This phenotype resembles that of *eaf6-2*, because under these conditions, some plants show a strong delay in flowering and some others are unable to flower. We hypothesized that this phenotype could be due to defects in the biosynthesis or the perception of the gibberellin signal, so we decided to test the effect on flowering of the exogenous addition of a mixture of bioactive GA (GA_{4+7}) to wt and *eaf6-2* plants grown in SD. We sprayed plants with this GA mixture once a week until they flowered, including a group of mock-treated plants as a control. We observed that, as expected, the treatment with GA accelerated the floral transition in Col plants (Fig. 15A, B). And even though this treatment did not rescue completely the late flowering phenotype of *eaf6-2* plants, it caused them to flower significantly earlier than mock-treated plants, indicating that *eaf6-2* plants are not affected in the response to GA (Fig. 15A, B). Conversely, the late flowering phenotype of these mutants might be caused, at least partially, by a deficient biosynthesis of GA, since exogenous GA can partially rescue this phenotype.

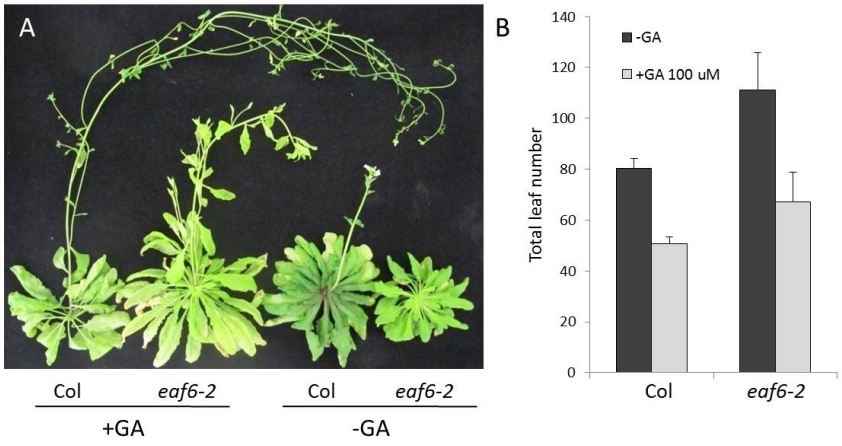

Figure 15. GA can partially rescue the late flowering phenotype of *eaf6-2* in SD. (A) Effect of GA_{4+7} treatment on the phenotype of Col and *eaf6-2* plants grown in SD. Plants treated with GA (+GA) were sprayed with a solution of 100 µM GA_{4+7} once a week until they flowered. Untreated Col and *eaf6-2* plants were used as a control (-GA). **(B)** Total leaf number at flowering of Col and *eaf6-2* plants grown in SD and treated with 100 µM GA_{4+7}. Untreated Col and *eaf6-2* plants were used as a control.

1.3.5. eaf6-2 FRI plants show a severe late flowering phenotype that is independent of FLC.

FLC is one of the main floral repressors in Arabidopsis and FRI is a potent activator of *FLC* expression. Plants with active alleles of *FRI* display a late flowering phenotype and elevated levels of *FLC* compared to *fri* plants (Crevillen and Dean, 2011). Mutations in components of the SWR1 complex, such as *ESD1/ARP6* or *SWC6* can suppress the late flowering phenotype of Col plants carrying active alleles of *FRI* from Sant Feliu background (Martin-Trillo et al., 2006; Lazaro et al., 2008). Given the possible relationship of SWR1-C and NuA4-C, and the decreased expression of *FLC* observed in mutants of Arabidopsis homologues of the NuA4-C, we hypothesized that this HAT complex might also be regulating flowering time through the regulation of *FLC*. In that case, changes in *FLC* expression and/or flowering time would be more easily noticeable in a *FRI* background. To test this, we introduced the *eaf6-2* mutation in a Col-*FRI* background and we analyzed flowering time and *FLC* expression in these plants. As shown in Fig. 16, *eaf6-2 FRI* plants showed a dramatic delay in flowering, to the point that only 4 plants out of 15 analyzed were able to flower, whereas the remaining 11 senesced and died before bolting. On average, the number of leaves produced by these plants was higher than 170, highlighting the severity of this phenotype (Fig. 16A, B). Thus, it is possible that this delay in flowering time could be caused by an upregulation of *FLC* in *eaf6-2 FRI* plants. To test this, we measured *FLC* expression levels by qPCR in Col, Col *FRI*, *eaf6-2 FRI* and *arp6 FRI* plants grown in LD for 8, 10 and 12 days. It has been described that mutations in *ARP6* are able to suppress the upregulation of *FLC* in *FRI* backgrounds (Martín-Trillo et al, 2006), so we used *arp6-1 FRI* plants as a control. In this case we did not detect significant changes in *FLC* expression in *eaf6-2 FRI* plants compared to Col *FRI* in any of the three time points analyzed. Altogether, these data indicate that the late flowering phenotype of *eaf6-2*

FRI plants is not mediated by *FLC*, and therefore, it might be due to the misregulation of other flowering genes.

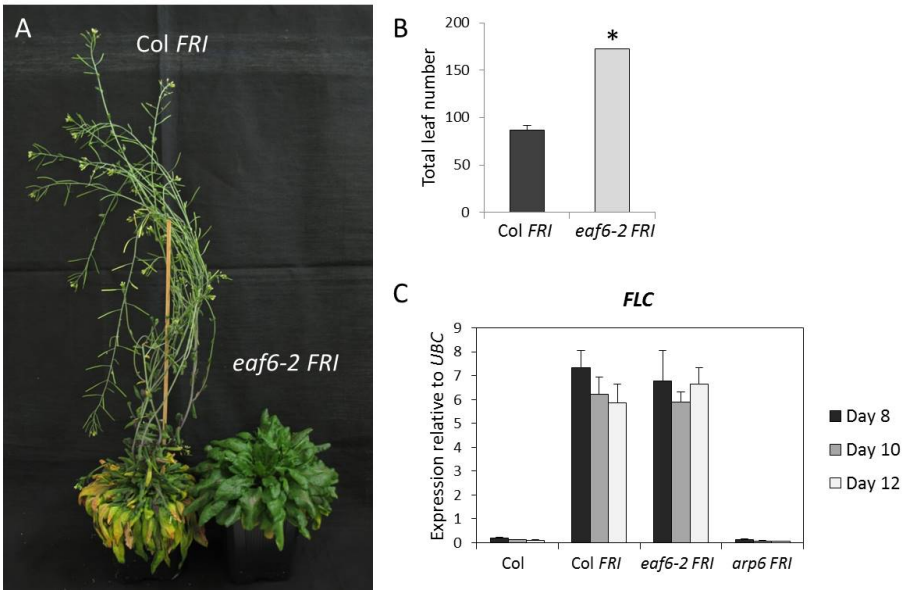

Figure 16. The *eaf6-2* mutation causes an *FLC*-independent delay of flowering in *FRI* background. (A) Flowering time phenotype of Col *FRI* and *eaf6-2 FRI* plants grown in LD. **(B)** Total leaf number at flowering in Col *FRI* and *eaf6-2 FRI* plants grown in LD. The asterisk (*) means that only 4 plants out of 15 were able to flower. **(C)** Expression of *FLC* in Col, Col *FRI*, *eaf6-2 FRI* and *arp6 FRI*, analyzed by qPCR in seedlings grown in LD for 8, 10 and 12 days. *UBC* expression was used as a control.

2. Characterization of MRG proteins in Arabidopsis

2.1. MRG1 and MRG2 proteins are the Arabidopsis homologues of yeast Eaf3

The *S. cerevisiae* NuA4 complex contains several subunits that harbor domains typically involved in interactions with chromatin, namely, Plant Homeo Domain (PHD) finger, SWI3, ADA2, *N*-CoR and TFIIIB (SANT), Yaf9, ENL, AF9, Taf14, Sas5 (YEATS), and chromodomain (CHD). The CHD is normally found in the form of three β strands packed against an α helix in a C-terminal position and it mediates protein-protein and/or protein-nucleic acid interactions, but its main role is acting as a reader of methylated K in the chromatin (Eissenberg, 2012). The yeast NuA4 complex encompasses two proteins bearing a CHD domain: the catalytic subunit Esa1, and Eaf3 (Doyon and Cote, 2004). The CHD in Esa1 has been shown to bind RNA (Shimojo et al., 2008), to play a key role in the ability of Piccolo to distinguish between histones and nucleosomes, and is essential for the HAT catalytic activity of Esa1 (Selleck et al., 2005). The CHD domain in Eaf3 mediates the binding of this protein to methylated H3K36 and H3K4, two epigenetic marks associated with active transcription. Four amino acid residues are essential for the binding of Eaf3 to methylated H3K36 in the CHD: Tyr23, Tyr81, Trp84 and Trp88. These residues form an aromatic cage, a cube-like structure that accommodates methylated H3K36, and mutations in these residues disrupt this association (Xu et al., 2008a; Sun et al., 2008).

S. cerevisiae Eaf3 belongs to the MRG family of proteins, which is highly conserved in fungi, plants and animals. Orthologues of Eaf3 are present in other yeast species (*Schizosaccharomyces pombe*), worms (*Caenorhabditis elegans*), flies (*Drosophila melanogaster*) and humans (*Homo sapiens*). Given the crucial role of MRG proteins in recognizing methylated H3K36, and the high degree of conservation of these proteins in different species, we decided to investigate the existence of MRG homologues in Arabidopsis and to analyze their role in the regulation of flowering.

To identify MRG orthologues in Arabidopsis, we searched the Arabidopsis genome for genes encoding proteins that share homology with Eaf3. We found two very similar proteins to the yeast counterpart, encoded by At4g37280 and At1g02740, which we designated as AtMRG1 and AtMRG2, respectively. To determine the degree of similarity of these proteins to different MRG proteins from other organisms, we ran an alignment to compare their protein sequences to the sequences of Eaf3 from *S. cerevisiae*, MRG15 from *D. melanogaster*, and MRG15 from *H. sapiens* (Fig. 17). We observed two regions that are highly conserved in all proteins analyzed: one towards the N-terminus that corresponds to the CHD domain (marked in red in Fig. 17), and the other one towards the C-terminus that corresponds to an MRG domain, predicted by PFAM (Fig. 17). The four amino acids involved in the recognition of methylated H3K36 by *S. cerevisiae* Eaf3 are well conserved in both Arabidopsis MRG1 and MRG2, as well as in the other EAF3 proteins analyzed (marked in blue in Fig. 17), suggesting that the role of these proteins in recognizing this important epigenetic mark might also be conserved in Arabidopsis.

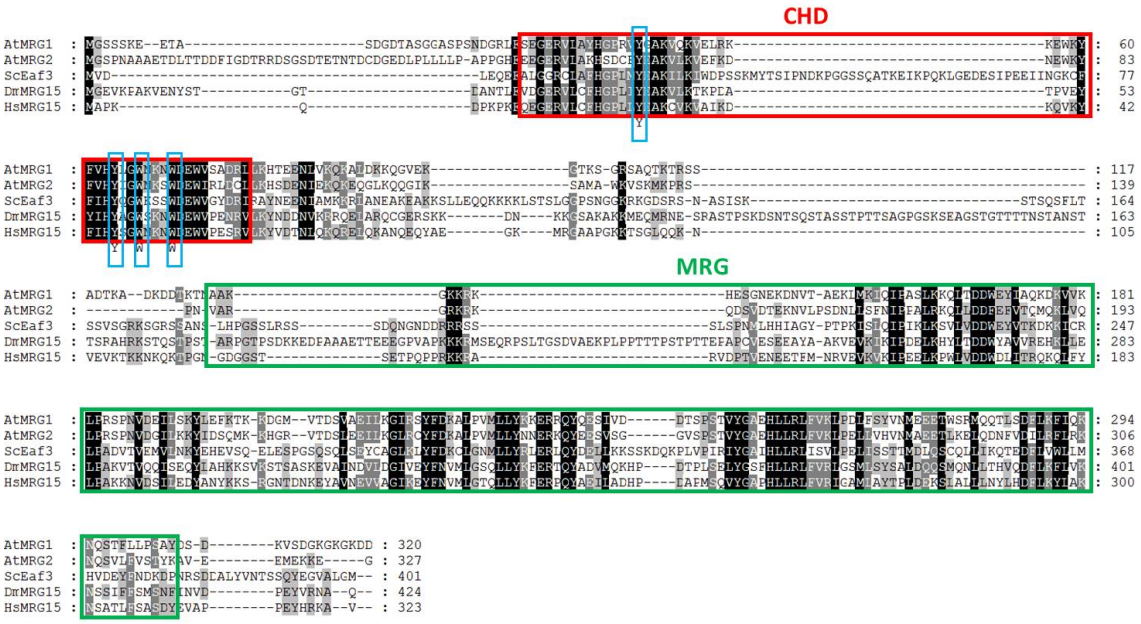

Figure 17. *AtMRG1* and *AtMRG2* encode evolutionary conserved chromodomain-containing proteins. Comparison of protein sequences of MRG proteins from *Arabidopsis thaliana* (AtMRG1 and AtMRG2), *Saccharomyces cerevisiae* (ScEaf3), *Drosophila melanogaster* (DmMRG15) and *Homo sapiens* (HsMRG15). Black, dark-gray, and light-gray shades represent conservation percentages of 100, 80, and 60, respectively. Residues in the red box form the conserved CHD and the four conserved amino acids that form the aromatic cage within the CHD responsible for the recognition of methylated H3K36 are marked in blue. Residues in the green box encompass the predicted MRG domain.

MRG1 and MRG2 protein sequences share a 46.56% of identity according to the CLUSTALW tool from EMBL-EBI (not shown). To find out if this identity correlates with structural similarity, we generated three-dimensional models of both proteins using the SWISS-PROT/TrEMBL tool (http://swissmodel.expasy. org/) described by Schwede et al. (2003), that predicts the structure of a given protein by homology modelling, assuming that two proteins with similar amino acid sequences will also have similar structures. This tool generates the structure of a protein of interest using the structure of a previously characterized protein that is similar in sequence as a template. MRG1 and MRG2 models have been generated based on the structure of the Mortality factor 4-like protein 1 from human (PDB ID: 2EFI). When we compared the models of MRG1 and MRG2, we observed that both proteins are very similar, as their structures are almost superposed (Fig. 18A). In fact, the root mean square deviation (RMS) for this alignment, that is, the distance between equivalent atoms in both structures, is as small as 2.21 Å, which indicates that both proteins are also structurally similar. We also found that the four amino acids that form the aromatic cage are arranged in a cube-like disposition that could accommodate the H3K36me3 mark, as it has been described in other organisms (Fig. 18A).

We also compared the predicted structure of MRG1 with the structure of *S. cerevisiae* Eaf3 (PDB ID: 2K3X). In this case we also found a high degree of similarity (Fig. 18B), with an even smaller RMS value of 1.72 Å. These data indicate that MRG1 and MRG2 are very similar proteins, both in sequence and in structure, and that they are the likely homologues of Eaf3 in Arabidopsis. Also, the fact that the essential amino acids for the formation of the structure

that recognizes methylated H3K36 are also present in the Arabidopsis proteins and in a proper disposition for this binding, suggests that their function as H3K36me3 readers might also be conserved.

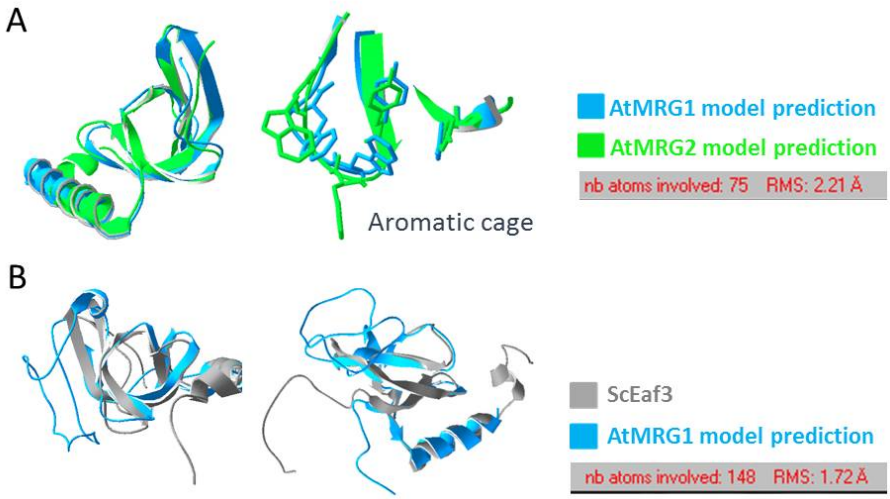

Figure 18. Arabidopsis MRG1 and MRG2 are structurally similar to each other and to yeast Eaf3. **(A)** Structural alignment of model structures predicted by SWISS-MODEL for AtMRG1 and AtMRG2, and close-up of the four amino acids that form the aromatic cage responsible for the recognition of H3K36me3. **(B)** Structural alignment of the model structure of AtMRG1 and *S. cerevisiae* Eaf3. The number of atoms involved in the alignment and the root mean square deviation (RMS) are shown for both alignments.

2.2. Characterization of loss-of-function mutants of MRG1 *and* MRG2

The Eaf3 homologues in Arabidopsis MRG1 and MRG2 are encoded by genes with 11 and 10 exons, respectively. To characterize the function of these two genes in the regulation of flowering time, we isolated one T-DNA insertion line for each of them (Fig. 19A), designated as *mrg1-2* and *mrg2-4*. The *mrg1-2* allele corresponds to the SALK_089867 line and it bears a T-DNA insertion in intron 6, whereas the *mrg2-4* allele corresponds to the SAIL_317_F11 line and harbors a T-DNA insertion in intron 4. We analyzed the expression of *MRG1* and *MRG2* in Col, *mrg1-2* and *mrg2-4* by qPCR. As shown in Fig. 19B, the *mrg1-2* and *mrg2-4* mutations completely abolish the expression of *MRG1* and *MRG2*, respectively, indicating that they are likely loss-of-function alleles. We also observed that the expression of *MRG2* was slightly elevated in the *mrg1-2* mutant, and *MRG1* was also slightly upregulated in the *mrg2-4* mutant, suggesting that the loss of function of each one of these genes causes an increase in the expression of its close homolog.

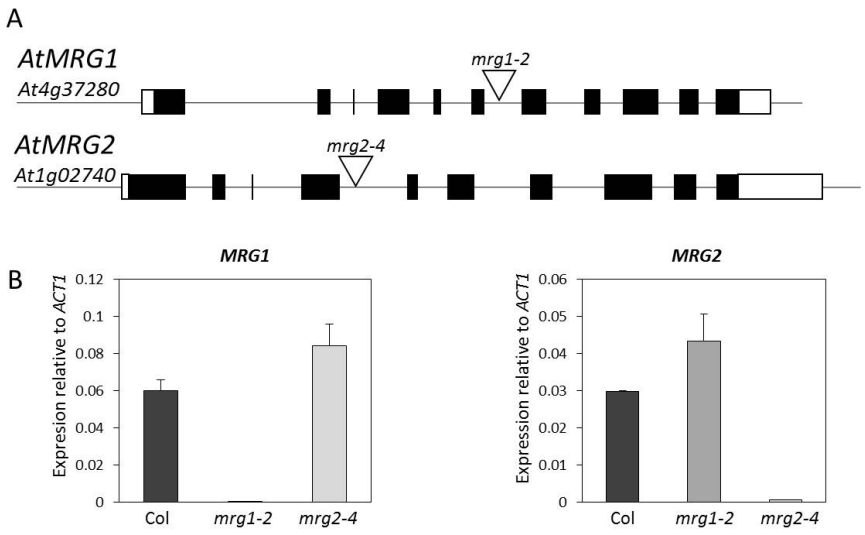

Figure 19. Isolation of loss-of function mutations for the Arabidopsis *MRG1* and *MRG2* genes. (A) Gene structure of *AtMRG1* and *AtMRG2* and location of the T-DNA in the insertion lines identified for these genes. Boxes symbolize exons and the black line represents introns. The white boxes represent the 5' and 3' UTRs (left and right, respectively). The inverted triangles indicate the position of the T-DNA insertions in the *mrg1-2* and *mrg2-4* alleles. **(B)** Expression of *MRG1* and *MRG2* in Col, *mrg1-2* and *mrg2-4,* analyzed by qPCR in 10-day-old seedlings grown in LD conditions. *ACT1* expression was used as a control.

2.3. MRG1 *and* MRG2 *play redundant roles in the control of flowering under LD*

To study the possible role of *MRG1* and *MRG2* in the regulation of flowering time, we analyzed the flowering phenotype of the *mrg1-2* and *mrg2-4* mutants under LD and SD. Under inductive LD conditions, we only found a slight delay in flowering in the *mrg1-2* mutant, whereas the phenotype of *mrg2-4* was indistinguishable from the wt (Fig. 20A, B). Under non-inductive SD conditions, we observed no significant differences between either the *mrg1-2* and the *mrg2-4* single mutants and the wt plants (Fig. 20C). To uncover any possible functional redundancy between *MRG1* and *MRG2* in the regulation of flowering time, we crossed the *mrg1-2* and *mrg2-4* mutants and generated *mrg1-2 mrg2-4* double mutants. We analyzed the flowering time of these plants under LD and we found that the *mrg1-2 mrg2-4* double mutant displayed a very late flowering phenotype (Fig. 20A, B). However, when we performed this experiment under SD, we could not detect significant differences between the double mutant and the wt plants. These results indicate that *MRG1* and *MRG2* act redundantly to regulate flowering time only under LD. Given that the delay in flowering time is only observed under this photoperiod, this suggests a possible involvement of the MRGs proteins in the photoperiodic pathway that controls flowering time.

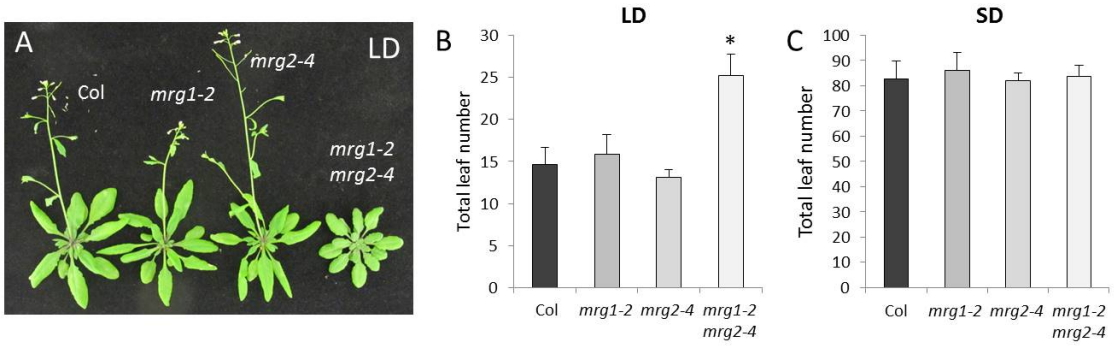

Figure 20. The double mutant *mrg1-2 mrg2-4* displays a late flowering phenotype only under LD. **(A)** Flowering time phenotype of Col, *mrg1-2, mrg2-4* and *mrg1-2 mrg2-4* plants under LD. **(B)** Total leaf number at flowering of Col, *mrg1-2, mrg2-4* and *mrg1-2 mrg2-4* plants grown in LD conditions. **(C)** Total leaf number at flowering of Col, *mrg1-2, mrg2-4* and *mrg1-2 mrg2-4* plants grown in SD conditions. *P < 0.01 with Student's *t*-test.

2.4. MRG1 and MRG2 positively regulate the expression of master regulators of flowering initiation

To elucidate the molecular bases of the late flowering phenotype observed in *mrg1-2 mrg2-4* double mutants under LD, we analyzed the expression of the master regulators of the floral transition *FLC*, *FT* and *SOC1* in these plants. We observed that, under these conditions, the expression of these three genes was severely reduced in *mrg1-2 mrg2-4* mutants, particularly in the case of the floral repressor *FLC* and the floral integrator *FT* (Fig. 21). Although *FLC* has an opposite role to *FT* and *SOC1* in the control of flowering, the fact that *FT* and *SOC1* act downstream of *FLC* to activate the floral transition might explain why these plants show such late flowering phenotype despite showing a strong downregulation of *FLC*.

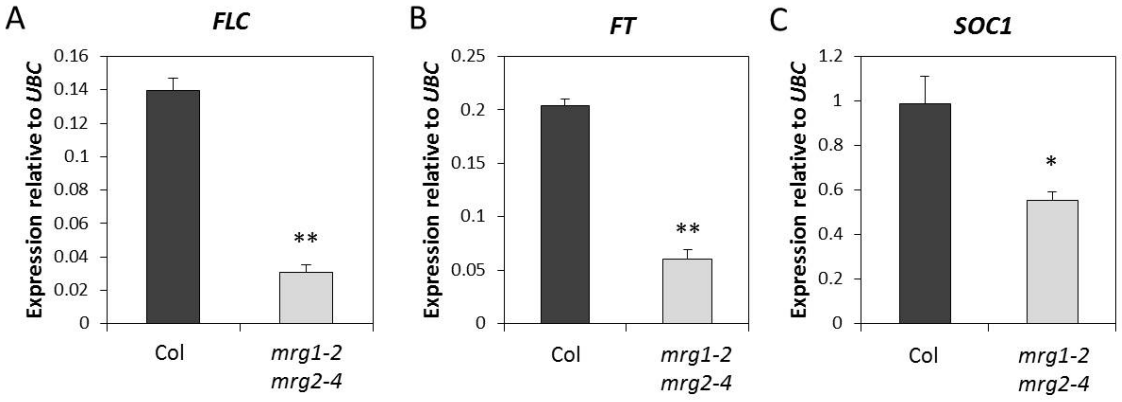

Figure 21. Expression of *FLC*, *FT* and *SOC1* in *mrg1-2 mrg2-4* under LD. Expression of *FLC* **(A)**, *FT* **(B)** and *SOC1* **(C)** in Col and *mrg1-2 mrg2-4*, analyzed by qPCR in 10-day-old seedlings grown in LD conditions . *UBC* expression was used as a control. *P < 0.05 with Student's t-test. **P < 0.01 with Student's t-test.

2.5. *Mutations in* FT *do not cause further delay in flowering time of* mrg1-2 mrg2-4 *plants*

The strong downregulation of *FT* observed in *mrg1-2 mrg2-4* plants might suggest a regulatory role of MRG proteins over this floral integrator gene. Previous reports had shown that the overexpression of *FT* fully rescues the late flowering phenotype of *mrg1-2 mrg2-4* plants, suggesting that MRG1 and MRG2 act upstream of *FT* (Bu et al, 2014). To corroborate this result, we crossed *mrg1-2 mrg2-4* and *ft-10* plants and generated an *mrg1-2 mrg2-4 ft-10* triple mutant. *ft-10* is a T-DNA insertional allele of *FT* that suppresses the early flowering phenotype of *35S::CO* plants. The flowering time of *mrg1-2 mrg2-4 ft-10* triple mutant plants was only slightly later compared to *ft-10* single mutants (Fig. 22: 67.54 leaves in *mrg1-2 mrg2-4 ft-10* to 60.5 leaves in *ft-10*), indicating that in the absence of a functional *FT*, the lack of MRG proteins can barely delay the floral transition, and therefore, that MRG proteins regulate flowering time by acting mainly upstream of *FT*.

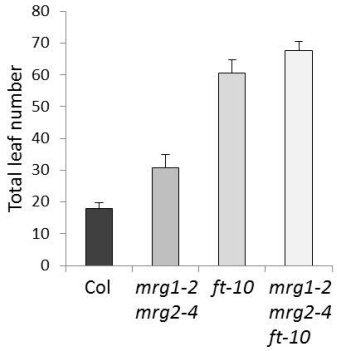

Figure 22. Flowering time phenotype of the *mrg1-2 mrg2-4 ft-10* triple mutant under LD. Total leaf number at flowering of Col, *mrg1-2 mrg2-4*, *ft-10* and *mrg1-2 mrg2-4 ft-10* plants grown under LD.

2.6. *Mutations in* SDG8 *fully suppress the late flowering phenotype of* mrg1-2 mrg2-4 *plants*

As mentioned above, Eaf3 acts as a reader of methylated H3K36 by binding di- and trimethylated H3K36 and recruiting chromatin remodeling complexes (Carrozza et al., 2005; Joshi and Struhl, 2005). Recent reports have shown that Arabidopsis MRG1 and MRG2 can also bind methylated H3K36 (Bu et al., 2014; Xu et al., 2014). In Arabidopsis, the most studied histone methyltransferase of the H3K36 residue is SET DOMAIN GROUP 8 (SDG8) protein, homologous to yeast H3K36 methyltransferase Set2 (Zhao et al., 2005). Mutations in *SDG8* lead to low levels of H3K36me3, reduced expression of *FLC* and early flowering (Zhao et al., 2005). To find out whether the function of *MRG1* and *MRG2* depends on SDG8-mediated H3K36 methylation, we generated *mrg1-2 mrg2-4 sdg8-1* triple mutants. We observed that these triple mutant plants displayed pleiotropic phenotypic alterations, showing dwarfism and extremely reduced fertility (Fig. 23A, B). The absence of *SDG8* also suppressed the late flowering phenotype of *mrg1-2 mrg2-4* plants, as the triple mutant flowered with approximately the same number of leaves as the *sdg8-1* single mutant. (Fig. 23A, C). These results indicate that, whereas *MRG1/2* and *SDG8* act additively to control several aspects of development, such as plant size and fertility, *SDG8* is epistatic over *MRG1/2* in the regulation of flowering time. The phenotype of the *mrg1-2 mrg2-4 sdg8-1* triple mutant indicates that the

occurrence of SDG8-mediated H3K36 methylation is necessary for the late flowering phenotype of *mrg1-2 mrg2-4* and suggests a functional relationship between both chromatin writer and reader activities.

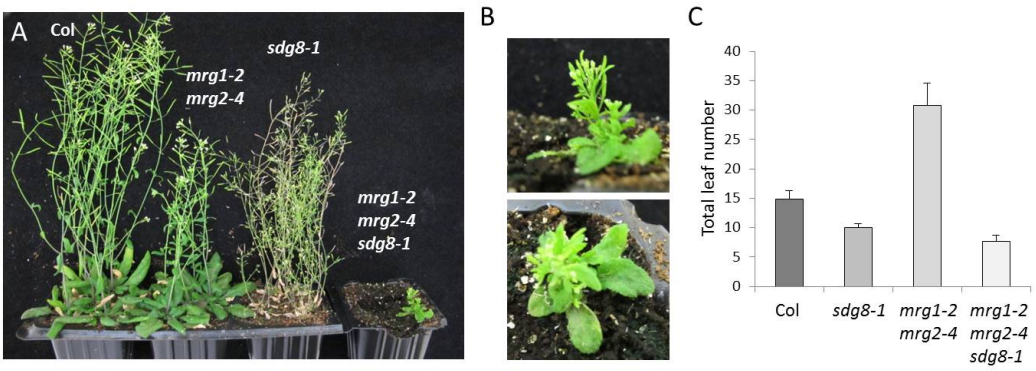

Figure 23. Mutations in *SDG8* suppress the late flowering phenotype of *mrg1-2 mrg2-4*. (A) Flowering time phenotype of Col, *mrg1-2 mrg2-4*, *sdg8-1* and *mrg1-2 mrg2-4 sdg8-1* plants in LD conditions. **(B)** Magnification of an *mrg1-2 mrg2-4 sdg8-1* plant. **(C)** Total leaf number at flowering of Col, *mrg1-2 mrg2-4*, *sdg8-1* and *mrg1-2 mrg2-4 sdg8-1* plants grown under LD.

2.7. MRG1 and MRG2 act additively with HDA6 to regulate flowering time

S. cerevisiae Eaf3 is shared by two chromatin remodeling complexes with antagonistic functions: the NuA4 HAT complex and the Rpd3 HDAC complex. HDA6 is a member of the RPD3/HDA1 family in Arabidopsis, and the *HDA6* mutant allele *axe1-5* exhibits a late flowering phenotype (Yu et al., 2011). Moreover, it has been shown that HDA6 interacts with FLD protein and that *axe1-5* shows increased expression of *FLC* (Wu et al., 2008; Yu et al., 2011). Given the functional relationship between Eaf3 and Rpd3 HDACs described in yeast and the similar flowering phenotypes exhibited by *axe1-5 and mrg1-2 mrg2-4* plants, we decided to investigate a possible genetic relationship between these loci. For that purpose, we generated the *mrg1-2 mrg2-4 axe1-5* triple mutant and analyzed its flowering time in LD. The triple mutant displayed an additive phenotype compared to the parental lines, as it flowered later than both *mrg1-2 mrg2-4* and *axe1-5* plants (Fig. 24). This result indicates that *MRG1/2* and *HDA6* regulate flowering through independent pathways.

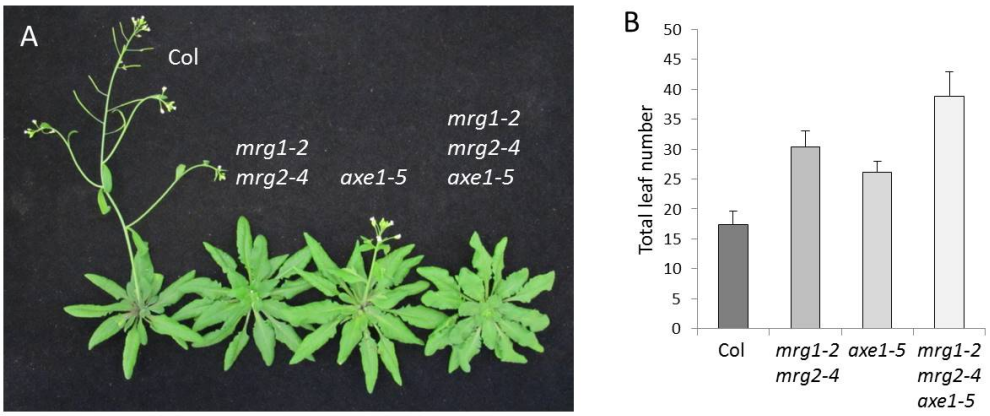

Figure 24. *MRG1* and *MRG2* act additively with *HDA6* to regulate flowering time. (A) Flowering time phenotype of Col, *mrg1-2 mrg2-4*, *axe1-5* and *mrg1-2 mrg2-4 axe1-5* plants in LD conditions. **(B)** Total leaf number at flowering of Col, *mrg1-2 mrg2-4*, *axe1-5* and *mrg1-2 mrg2-4 axe1-5* plants grown under LD.

2.8. MRG2 binds FLC *chromatin.*

Previous studies have shown that MRG proteins regulate *FT* directly, by binding to the chromatin of this floral integrator gene and modifying its levels of histone acetylation (Bu et al., 2014; Xu et al., 2014). The severe downregulation of *FLC* observed in *mrg1-2 mrg2-4* plants suggests that the regulation of MRG proteins over *FLC* might also be direct. To test this hypothesis, we analyzed the binding of MRG2 to the chromatin of *FLC* through ChIP experiments. We used a *pMRG2::MRG2-YFP* transgenic line that fully complements the flowering phenotype of *mrg1 mrg2* plants (Bu et al., 2014). Using an anti-GFP antibody, we analyzed the binding of MRG2 to three different regions of *FLC*: one in the promoter region (*FLC1*), another one next to the transcription start site (*FLC3*), and another one in the first intron (*FLC5*) (Fig. 25A). We also used a region in the *ACT2* gene as a genomic control for the ChIP experiments.

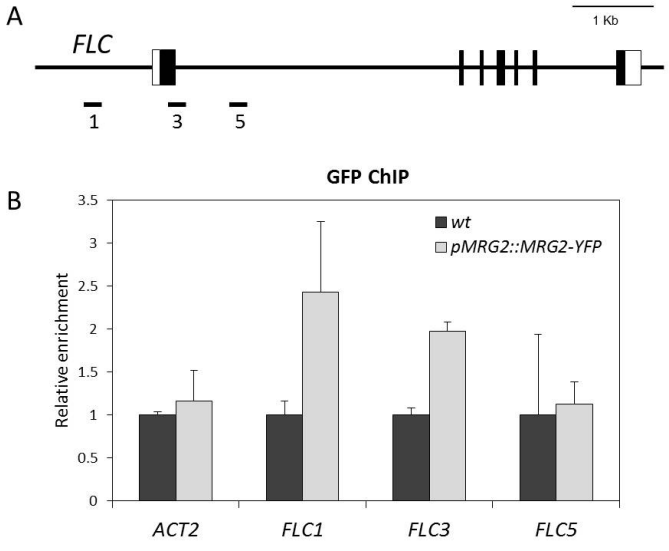

Figure 25. MRG2 binds the chromatin of *FLC*. (A) Representation of the Arabidopsis *FLC* gene and the regions amplified by qPCR. **(B)** ChIP analysis of MRG2-YFP enrichment at the *FLC* locus with a pMRG2::MRG2-YFP line versus wt plants. The amount of immunoprecipitated genomic fragments was measured by qPCR and referenced to the initial amount present in the input sample. Values in wt plants were set to 1. Error bars indicate SD of two biological replicates.

We observed no significant binding to *ACT2*; however, we detected a considerable enrichment of immunoprecipitated DNA in the *FLC1* and *FLC3* regions in the transgenic line compared to the non-transformed wt plants (Fig. 25B), indicating that MRG2 binds the chromatin of *FLC* in those regions, and suggesting that the reduction of *FLC* expression in *mrg1-2 mrg2-4* plants might be caused by a direct regulation of MRG proteins over *FLC*.

2.9. Analysis of histone mark distribution at FLC *in* mrg1-2 mrg2-4 *plants*

A previous report has shown that MRG2 is able to interact with the HATs HAM1 and HAM2, and that MRG proteins are necessary to maintain high levels of the H4K5Ac mark in the chromatin of another MRG2 direct target, the floral integrator *FT* (Xu et al., 2014). This suggests that MRG proteins might regulate gene expression by modulating the acetylation status in the chromatin of their target genes. To test if this was also the case in *FLC*, we analyzed the levels of H3 and H4 acetylation at *FLC* in wt and *mrg1-2 mrg2-4* plants by ChIP. We used antibodies that recognize H3 residues methylated at K9 and K14, and antibodies the recognize H4 tetra-acetylated at K5, K8, K16 and K20. We analyzed 8 different regions spanning the whole gene, from the promoter to the last exon (Fig. 26A). Data were normalized to the internal control *ACT2*. We observed that the distribution of acetylated H3 and H4 at *FLC* show very similar patterns (Fig. 26B, C). Both marks are highly enriched near the TSS (*FLC3* region) and moderately increased towards the end of the locus, in the last exon (region *FLC10*). We also observed that H3 acetylation levels were only slightly increased in *mrg1-2 mrg2-4* compared to the wt, indicating that the absence of MRG1/2 does not have major effects in the levels of this mark. We also detected a more pronounced increase in the levels of acetylated H4 in the double mutant in the *FLC3* region. These results indicate that the *mrg1-2 mrg2-4* mutations provoke a moderate increase in the levels of histone acetylation at *FLC* chromatin, especially in the case of acetylated H4, indicating that MRG1/2 might participate in or be necessary for a mechanism involving histone deacetylation, rather than histone acetylation at *FLC* chromatin. The increased levels of histone acetylation at *FLC* detected in the double mutant do not correlate with the reduced expression of this gene observed in these plants, and therefore, we hypothesized that the distribution of other histone marks, such as trimethylation of H3K4 and/or H3K27, might be altered. The H3K4me3 mark is found in actively transcribed genes (Santos-Rosa et al., 2002) and generally enriched in the promoters and 5' genic regions (Zhang et al., 2009), whereas H3K27me3 is associated with repressed genes (He and Amasino, 2005; Ringrose and Paro, 2007). To assess if the mutations in *MRG1* and *MRG2* caused alterations in the abundance of these two histone marks at *FLC* chromatin, we analyzed the distribution of both marks along this locus in Col and *mrg1-2 mrg2-4* plants by ChIP, using specific antibodies.

We found that the distribution of H3K4me3 shows a peak in the *FLC3* region, consistently with previous reports (Tamada et al., 2009; Yang et al., 2014). We observed no significant changes in the abundance of this mark in the *mrg1-2 mrg2-4* double mutant, indicating that MRG proteins are not involved in its deposition (Fig. 26D). H3K27me3 showed a broader distribution along the locus, also in agreement with previous studies (De Lucia et al, 2008; Yang et al, 2014). In this case, we found a reduction in the levels of this mark in many of

the regions analyzed in the *mrg1-2 mrg2-4* double mutant (Fig. 26E), suggesting that MRG proteins might be positively regulating the deposition of H3K27me3. However, these reduced levels of H3K27me3 could not either explain the reduced expression of *FLC* observed in the *mrg1-2 mrg2-4* plants.

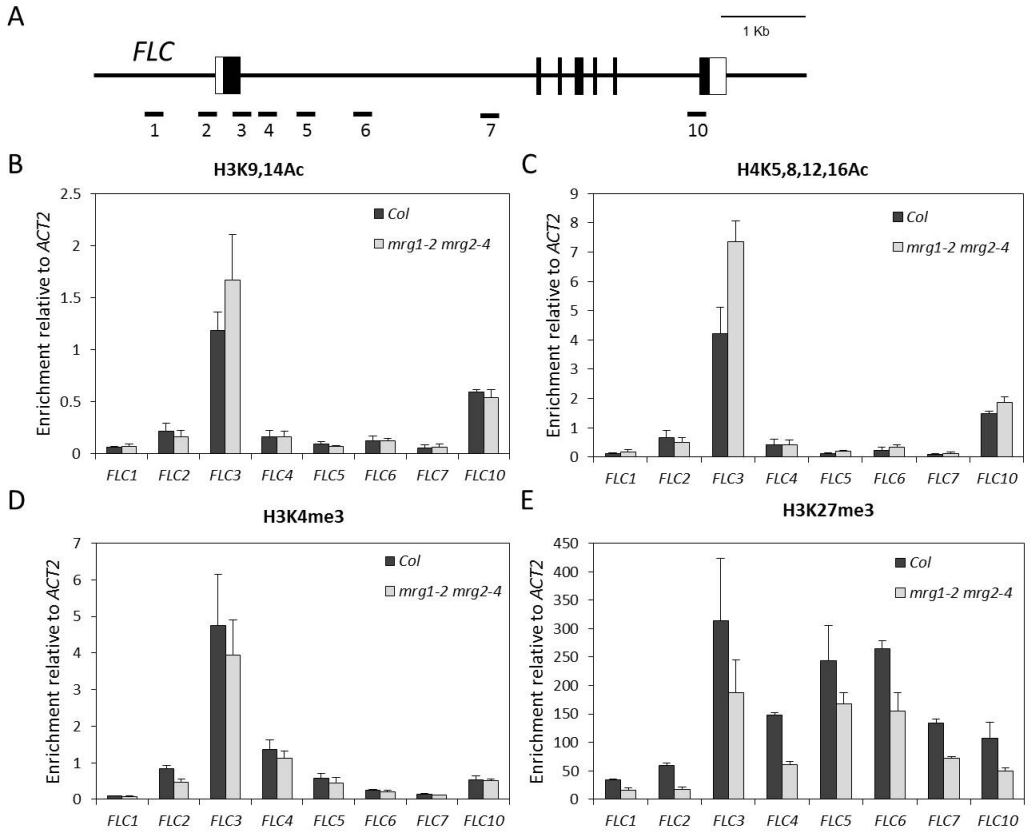

Figure 26. Analysis of histone mark distribution at *FLC* in *mrg1-2 mrg2-4* plants. (A) Representation of the Arabidopsis *FLC* gene and the regions amplified by qPCR. ChIP analyses of H3K9,14Ac **(B)**, H4K5,8,16,20Ac **(C)**, H3K4me3 **(D)**, and H3K27me3 **(E)** distribution along the *FLC* locus in Col and *mrg1-2 mrg2-4* plants. The amount of immunoprecipitated genomic fragments was measured by qPCR and enrichment was calculated by normalizing the values of immunoprecipitated DNA to inputs and to an internal control (*ACT2*). Error bars indicate SD of three biological replicates.

2.10. Mutations in MRG1 *and* MRG2 *do not affect the deposition of H2A.Z at* FLC *chromatin.*

The SWR1 complex in Arabidopsis regulates flowering by exchanging histone H2A by the histone variant H2A.Z. This activity is required for the transcriptional activation of the *FLC* gene (Lazaro et al., 2008; Deal et al., 2007). Yeast SWR1 shares four subunits with NuA4 complex and the activity of NuA4 complex stimulates SWR1-mediated deposition of H2A.Z (Altaf et al., 2010). Given this functional relationship, we wondered whether the downregulation of *FLC* observed in *mrg1-2 mrg2-4* plants could be due to impaired H2A.Z deposition in this locus. To analyze the distribution of H2A.Z at *FLC*, we used a transgenic line expressing the H2A.Z gene *HTA11* fused to *GFP* under the control of its own promoter (*pHTA11::HTA11-GFP*), which complements the phenotype of *hta9 hta11* double mutant plants

(Kumar and Wigge, 2010). We introgressed this transgene into the *mrg1-2 mrg2-4* background and performed ChIP experiments with an anti-GFP antibody to follow the occupancy of the H2A.Z encoded by *HTA11* at the *FLC* locus in Col and *mrg1-2 mrg2-4* backgrounds. We used Col non-transformed plants as a negative control. H2A.Z distribution peaks at the 5' and the 3' end of the *FLC* locus, and the H2A.Z levels are lower at the end of the first intron (Deal et al., 2007). Therefore, we decided to analyze H2A.Z occupancy in three regions of *FLC*: at the 5' end (*FLC3*), at the first intron (*FLC7*) and at the 3' end (*FLC10*) (Fig. 27A). We also assessed a region of At4g07700 that does not contain H2A.Z (Zilberman et al., 2008) as a negative control.

The results of this experiment show that, as expected, HTA11 levels in a Col background are elevated in *FLC3* and *FLC10* regions, low at *FLC7*, and almost undetectable at At4g07700. Moreover, we could not find significant differences in the occupancy of HTA11 H2A.Z in any of the regions tested in the *mrg1-2 mrg2-4* background compared to the wt (Fig. 27B), which suggests that the absence of MRG1 and MRG2 does not impair the deposition of H2A.Z mediated by SWR1 complex at *FLC* and therefore, the reduced levels of *FLC* expression in this double mutant are not due to a defective deposition of H2A.Z at this locus.

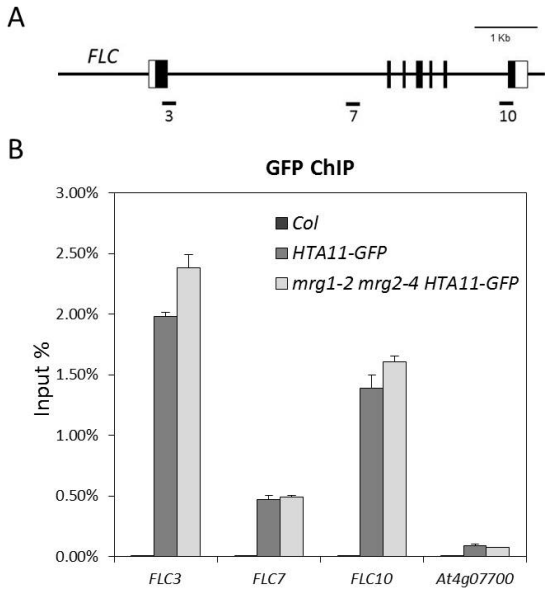

Figure 27. Mutations in *MRG1* and *MRG2* do not affect H2A.Z deposition at the *FLC* locus. (A) Representation of the Arabidopsis *FLC* gene and the regions amplified by qPCR. **(B)** Enrichment of H2A.Z at the *FLC* locus analyzed by ChIP using an anti GFP antibody in HTA11-GFP lines in Col and *mrg1-2 mrg2-4* backgrounds. Col plants were used as a negative genetic control. The amount of immunoprecipitated genomic fragments was measured by qPCR and expressed as input %. A region of the At4g07700 that does not contain H2A.Z was used as a negative genomic control. Error bars indicate SD of a representative experiment.

2.11. Transcriptomic analysis of mrg1-2 mrg2-4

To determine potential candidate genes regulated by MRG1 and MRG2, we decided to perform a transcriptomic analysis by RNA seq with the *mrg1-2 mrg2-4* double mutant. We extracted RNA from samples collected at ZT16 from 10-day-old Col and *mrg1-2 mrg2-4* seedlings grown in LD. Each sample was collected in triplicate, and the data from the *mrg1-2 mrg2-4* double mutant were compared to those from Col plants.

We found 1084 misregulated genes in *mrg1-2 mrg2-4*, 785 of which were upregulated and 299 were downregulated. A heatmap diagram representing the 20 genes that were more up- and downregulated in *mrg1-2 mrg2-4* compared to the wt is shown in Fig. 28. The gene displaying the highest up-regulation level was At3g01329, which encodes an uncharacterized ECA-1-like gametogenesis related family protein. Among other upregulated genes, we can find

HR2, an homologue of the broad-spectrum mildew resistance gene *RPW8.2* (Xiao et al., 2004), *HEME OXYGENASE 4* (*HO4*), which is involved in the biosynthesis of phytochrome chromophore and plays a role in photomorphogenesis (Emborg et al., 2006) and *LATERAL ORGAN BOUNDARIES* (*LOB*), that limits growth in organ boundaries by negatively regulating brassinosteroid accumulation (Bell et al., 2012). We can also observe that *PHERES1*/*AGL37*, which encodes a transcription factor involved in embryo development (Villar et al., 2009; Schmidt et al., 2013)and *JASMONATE ZIM-DOMAIN 3* (*JAZ3*), encoding a repressor of jasmonic acid responses (Chini et al., 2007) are also among the top 20 most upregulated genes in *mrg1-2 mrg2-4* seedlings.

The most downregulated gene was *PLANT DEFENSIN 1.2C*, (*PDF1.2C*), and we can observe that other members of this family are also among the most downregulated genes in this double mutant, like *PDF1.2A* and *PDF1.2B*. The glutathione-S-transferases GSTU3 and GST11 were also downregulated, as well as the salt-responsive *PUMILIO 21* (*PUM21*) and the cold-induced *GLYCINE-RICH PROTEIN 8* (*GRP8*) genes.

Consistent with the delay in the time of flowering displayed by the double mutant, the expression of the floral integrator gene *FT* was also significantly downregulated in this mutant, corroborating our previous results (section 2.4). Accordingly, the expression of the floral repressors *TEMPRANILLO 1* and *2 (TEM1* and *2)* was upregulated.

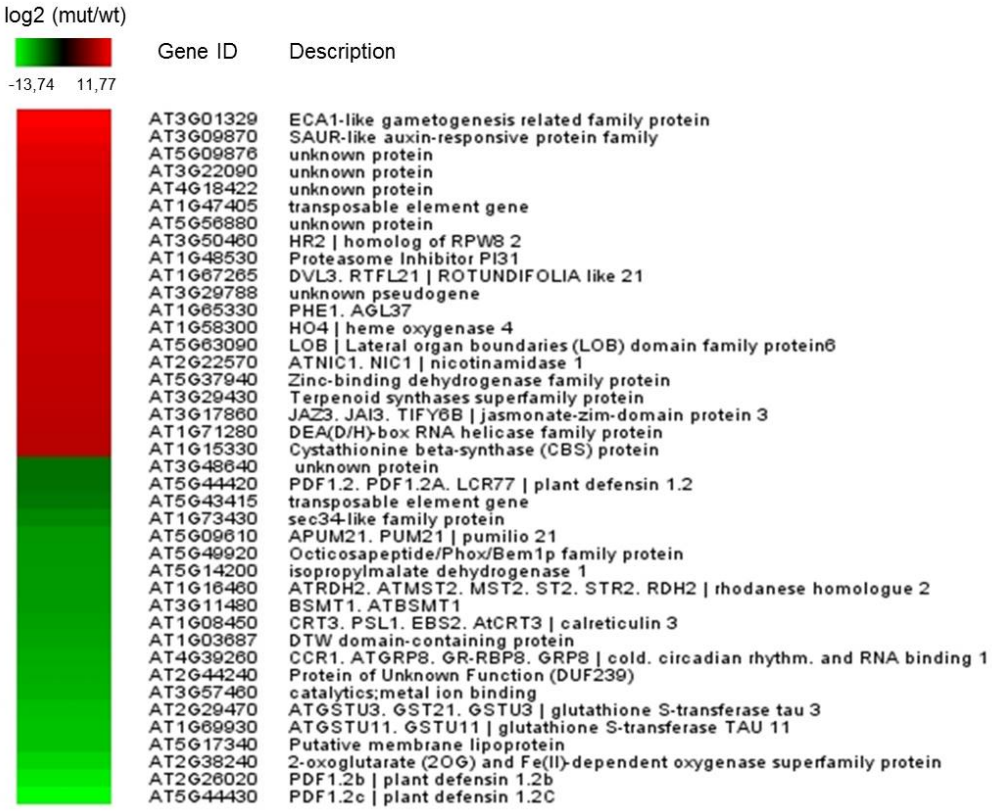

Figure 28. Genes differentially expressed in *mrg1-2 mrg2-4* seedlings. Shown are the 20 genes more upregulated and downregulated, respectively. From left to right: expression values expressed as log2 (mut/wt), gene identificator (AGI number) and description of each gene.

To validate the results obtained in the transcriptomic analysis of *mrg1-2 mr2-4* plants, we selected some of the genes that showed altered expression in *mrg1-2 mrg2-4* seedlings and checked their expression by qPCR. The *JAZ7* and *TEM2* genes appeared to be upregulated in our RNAseq data. We confirmed their increased expression in *mrg1-2 mrg2-4* seedlings (Fig. 29A, B). In contrast, *TYROSINE AMINOTRANSFERASE 3* (*TAT3*), *PATHOGENESIS-RELATED GENE 1* (*PR1*) and *EXPANSIN A1* (*EXPA1*) were downregulated in the RNAseq data. We were also able to confirm the expression changes in those genes by qPCR (Fig. 29C, D, and E). This analysis confirms the results obtained from the RNAseq experiments and corroborate the misregulation of these genes in *mrg1-2 mrg2-4* double mutant plants. These data are consistent with MRGs proteins acting as key regulators of gene expression in Arabidopsis, and anticipates additional roles for these proteins in various aspects of plant biology.

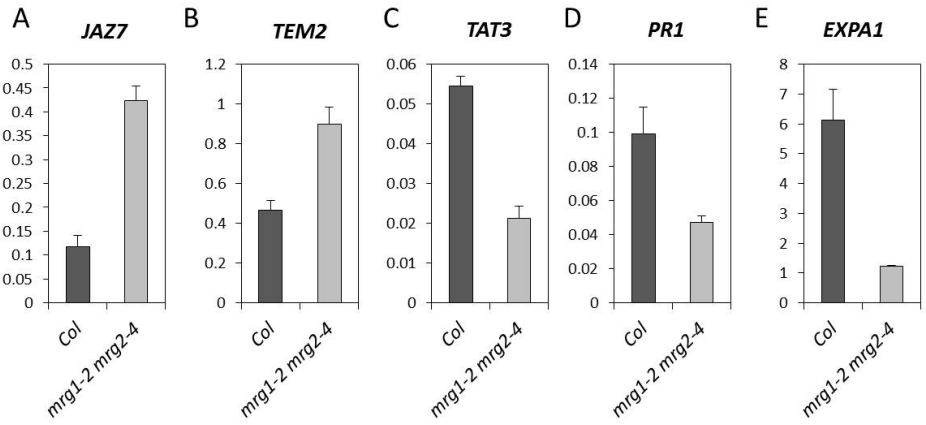

Figure 29. Expression analysis of misregulated genes in *mrg1-2 mrg2-4* plants. Expression of *JAZ7* **(A)**, *TEM2* **(B)**, *TAT3* **(C)**, *PR1* **(D)** and *EXPA1* **(E)** in Col and *mrg1-2 mrg2-4*, analyzed by qPCR in 10-day-old seedlings grown in LD. *UBC* expression was used as a control.

We carried out a GO analysis among up- and down-regulated genes attending to biological process and molecular function (Fig. 30). We observed that, regarding the biological process, there is a significant enrichment of genes involved in transcriptional regulation among the up-regulated genes. Response to chitin, response to abscisic acid stimulus and response to wounding are categories that also show enrichment among the upregulated genes. Among the down-regulated genes we find a large enrichment in defense response, response to water deprivation and to jasmonic acid stimuli genes. The molecular function GO analysis uncovered a strong enrichment in genes with transcription factor and DNA binding activities among the up-regulated genes, and kinase-related activity among the downregulated genes (Fig. 30). This indicates that MRG1 and MRG2 are directly or indirectly involved in the regulation of a diverse array of biological processes, and the high enrichment in transcription factors and DNA-binding factors found among the misregulated genes highlights the crucial regulatory role played by these proteins in Arabidopsis development.

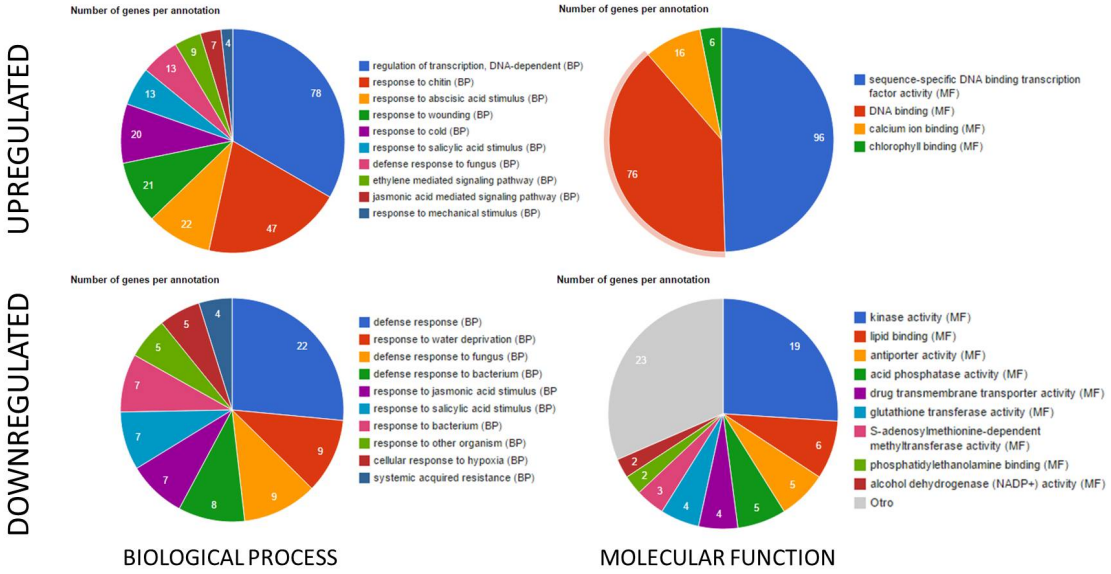

Figure 30. Enrichment of GO functional categories among up-regulated and down-regulated genes in *mrg1-2 mrg2-4* mutant plants. Number of genes per category attending to biological process **(A, C)** and molecular function (B, D) among upregulated **(A, B)** and downregulated **(C, D)** genes in *mrg1-2 mrg2-4*.

3. Characterization of Arabidopsis *ING1* and *ING2*

3.1. Functional analysis of AtING1 and AtING2

3.1.1. AtING1 *and* AtING2 *encode members of the PHD-containing ING family of proteins*

Among the *S. cerevisiae* NuA4 components with chromatin-interacting domains, the Yng2 subunit plays an important role in mediating the interaction of the Piccolo subcomplex with the nucleosomes and positioning it in an orientation that places the histone tails in a location close to the Esa1 catalytic site (Chittuluru et al., 2011). Yng2 belongs to the ING family of proteins, which is characterized by bearing a C-terminal PHD finger (Shi and Gozani, 2005; Aguissa-Toure et al., 2011). The conserved PHD domain fold is formed by two antiparallel β-strands and a C-terminal α-helix. Two zinc atoms fixed by a Cys_4-His-Cys_3 double loop organized in a cross-brace topology stabilize the structure (Sanchez and Zhou, 2011). PHD fingers are typically involved in the recognition of methylated lysines in the chromatin, and PHD-containing proteins usually act as "nucleosome readers" that mediate transcriptional activation or repression of underlying genes (Mouriz et al., 2015). Most characterized PHD-proteins recognize H3K4me3, a landmark of transcriptionally active genes. However, PHD-mediated recognition of other histone marks has also been reported (Li and Li, 2012; Musselman and Kutateladze, 2011). Structural studies have unveiled the determinants required for the recognition of target histone modifications by PHD fingers (Sanchez and Zhou, 2011). The recognition of methylated H3K4 is mediated by an aromatic cage on the surface of one β–sheet that accommodates the methylated peptide, and is usually composed by two to four aromatic residues (Musselman and Kutateladze, 2011). ING family members can be present in HAT or HDAC complexes and therefore, the recognition of their target histone marks can result in either transcriptional activation or repression (Doyon et al., 2006).

Given the prominent role of ING proteins in the chromatin-mediated regulation of transcription, and the role of Yng2 in the context of the yeast NuA4 complex, we pursued the identification and characterization of ING members in Arabidopsis. ING proteins are conserved in many eukaryotic organisms, from yeast to invertebrates, vertebrates including humans, and also in plants (He et al., 2005). In *S. cerevisiae* two members of this family are present: Yng1, which is part of the NuA3 HAT complex and Yng2, member of the NuA4-C However, the human genome contains up to five members of this family, ING1-5, which have been characterized and found to be part of different chromatin remodeling complexes and to be involved in many important cellular processes, such as apoptosis induction, DNA damage repair, control of cell cycle, etc. (Aguissa-Toure et al., 2011; Coles and Jones, 2009). To identify and characterize putative ING proteins in Arabidopsis that might be part of the NuA4 complex, we searched for proteins that share homology with ScYng2 in the Arabidopsis genome. We found two homologues encoded by At3g24010 and At1g54390 named as *AtING1* and *AtING2*, respectively (Lee et al., 2009). To determine the degree of similarity of these proteins to ING proteins from other organisms, we compared them with the sequences of Yng1 and Yng2 from *S. cerevisiae*, and ING1, ING2, ING3, ING4 and ING5 from *Homo sapiens* (Fig. 31). We

determined three regions showing a high degree of conservation, two of them in the N-terminal part, and the most conserved one towards the C-terminal end, which corresponds to the PHD domain. As shown in the Fig. 31, the residues required for the formation of the Cys_4-His-Cys_3 double loop are present in AtING1 and AtING2. We also observed that the key residues for the formation of the aromatic cage involved in the recognition of methylated H3K4 are fully conserved in the Arabidopsis proteins: Tyr, Met, and Trp. The high degree of similarity with ING proteins from other organisms indicates that AtING1 and AtING2 are indeed members of the ING family of proteins. Also, the fact that the key residues for the formation of the PHD finger and the aromatic cage are conserved in these proteins is consistent with previous reports that provided evidence supporting that the PHD fingers of ING1 and ING2 recognize H3K4me3, and therefore act as chromatin "readers" in Arabidopsis (Lee et al., 2009).

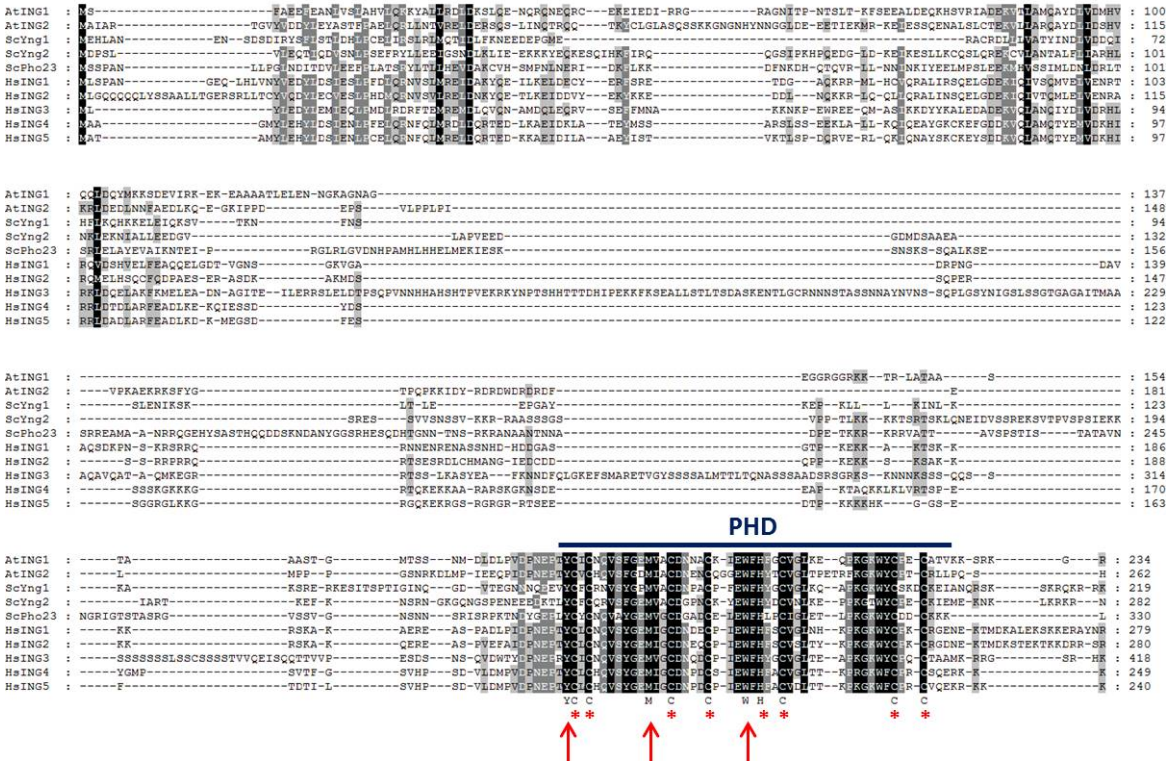

Figure 31. *AtING1* and *AtING2* encode evolutionary conserved PHD-containing proteins. Comparison of protein sequences of ING proteins from *Arabidopsis thaliana* (AtING1 and AtING2), *Saccharomyces cerevisiae* (ScYng1 and ScYng2), and *Homo sapiens* (HsING1, HsING2, HsING4 and HsING5). Black, dark-gray, and light-gray shades represent conservation percentages of 100, 80, and 60, respectively. Residues predicted to form the PHD finger are marked in dark blue. The Cys_4-His-Cys_3 residues required for the formation of the PHD finger are marked with red asterisks. Residues predicted to form the aromatic cage are indicated with red arrows.

3.1.2. Arabidopsis ING1 *is involved in the repression of the floral transition*

To characterize the function of ING1 in the regulation of the floral transition, we searched public databases for T-DNA insertion mutants for this gene. We identified a line (SALK_09598) that harbors a T-DNA insertion in exon 3 of this locus, and we designated it as *ing1-1* (Fig. 32A). To find out whether the *ing1-1* mutation leads to a decrease in the

expression of *ING1*, we analyzed the expression of this gene in the *ing1-1* mutant by qPCR. Using primers both upstream (*ING1 UPSTREAM*) and downstream (*ING1 DOWNSTREAM*) the T-DNA insertion (Fig. 32A), we observed that *ING1* expression in *ing1-1* plants is reduced to approximately 20% of the levels observed in wt plants, although it is not completely abolished. We also analyzed the expression of *ING1* in mutants deficient for the *ING2* homologue (*ing2-1*, discussed later) and we observed that mutations in *ING2* cause only a minor increase in the expression of its close homologue. In the rest of this study, *ING1* expression has been analyzed with the *ING1 UPSTREAM* primer pair.

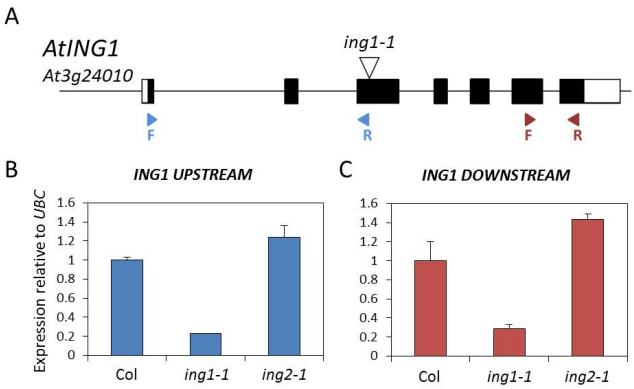

Figure 32. The *ING1* gene in *Arabidopsis thaliana* and insertion allele isolated. (A) Gene structure of *AtING1* and T-DNA insertion line. Boxes represent exons and the black line represents the introns. The white boxes represent the 5' and 3' UTRs (left and right, respectively). The inverted triangle represents the position of the T-DNA insertion in the *ing1-1* allele. Arrows represent the position of the forward (F) and reverse (R) primers used to determine the expression of *ING1* upstream (blue) and downstream (red) the T-DNA insertion **(B)** Expression of *ING1* in Col, *ing1-1* and *ing2-1,* analyzed by qPCR using primers upstream the T-DNA insertion in *ing1-1*. **(C)** Expression of *ING1* in Col, *ing1-1* and *ing2-1,* analyzed by qPCR using primers downstream the T-DNA insertion in *ing1-1.* In **(B)** and **(C)**, expression of *ING1* was analyzed in 10-day-old seedlings grown under LD conditions. *UBC* expression was used as a control.

Next, we asked if mutations in *ING1* caused any alterations in flowering time. To answer this, we measured flowering time in Col and *ing1-1* plants both in LD and SD photoperiods. We observed that under LD, *ing1-1* plants flowered earlier than the wt (Fig 32A, B), whereas under SD, their flowering time was indistinguishable from the wt (Fig. 33C). This indicates that *ING1* is required to repress the floral transition under LD.

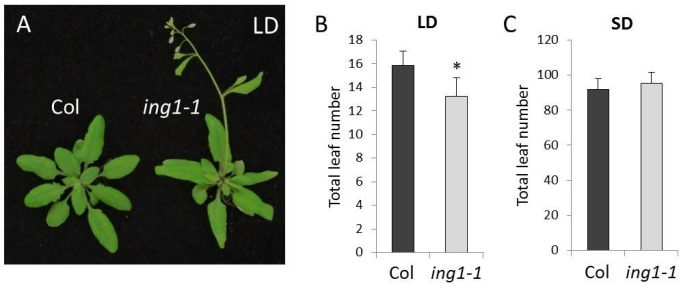

Figure 33. Mutations in *AtING1* cause an early flowering phenotype under LD. (A) Flowering time phenotype of *ing1-1* plants under LD. **(B)** Total leaf number at flowering of Col and *ing1-1* plants grown in LD conditions. **(C)** Total leaf number at flowering of Col and *ing1-1* plants grown in SD conditions.

To confirm that the alterations in flowering time observed in *ing1-1* mutant plants were caused by the mutation in *ING1*, we decided to complement this mutant with the corresponding wt *ING1* gene. For this purpose, we generated a *35S::FLAG-ING1* construct that was transformed into an *ing1-1* mutant background. We produced a number of independent transgenic lines and analyzed their flowering time, confirming that several lines showed a flowering phenotype similar to the wt (Fig. 34A, B). We also examined the expression of *ING1* in these lines and we confirmed that the transgenic plants showed a strong overexpression of the gene, reaching levels up to 40 times higher than the wt. Since the *35S::FLAG-ING1* construct can complement the early flowering phenotype of *ing1-1* in LD, we concluded that this phenotype is indeed caused by the loss of function of *ING1*.

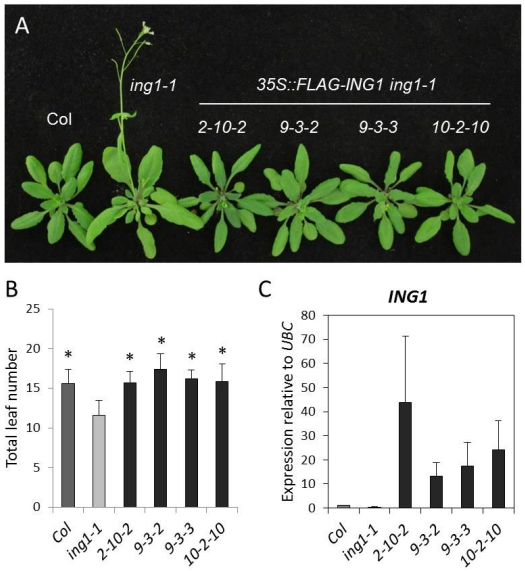

Figure 34. A *35S::FLAG-ING1* construct complements the early flowering phenotype of *ing1-1*. (A) Flowering time phenotype of Col, *ing1-1*, and the *35S::FLAG-ING1* lines *2-10-2*, *9-3-2*, *9-3-3* and *10-2-10* grown under LD. **(B)** Total leaf number at flowering of Col, *ing1-1* and the *35S::FLAG-ING1* lines *2-10-2*, *9-3-2*, *9-3-3* and *10-2-10* grown under LD. **(C)** Expression of *ING1* in Col, *ing1-1* and the *35S::FLAG-ING1* lines *2-10-2* and *10-2-10,* analyzed by qPCR in 10-day-old seedlings grown under LD conditions . *UBC* expression was used as control. Values in Col were set to 1. *P < 0.01 with Student's t-test when compared with ing1-1.

3.1.3. ING1 *is required to regulate the expression of master regulators of flowering*

To elucidate the molecular bases of the early flowering phenotype observed in *ing1-1* plants, we measured in Col and *ing1-1* plants the expression of several genes that have a prominent role in the regulation of flowering, such as the floral repressor *FLC*, the FLC-clade genes *MAF1*/*FLM*, *MAF2*, *MAF4* and *MAF5*, and the floral integrators *FT* and *SOC1* (Fig. 35). We observed a reduced expression of the floral repressors *FLC*, *MAF1*/*FLM*, *MAF2*, *MAF4* and *MAF5*, that was particularly conspicuous in the case of *FLC* and *MAF4*. Furthermore, we observed that *FT* and *SOC1* expression was upregulated in *ing1-1*. These results indicate that *ING1* participates in the regulation of the expression of these genes and are consistent with the early flowering phenotype observed in the *ing1-1* mutant.

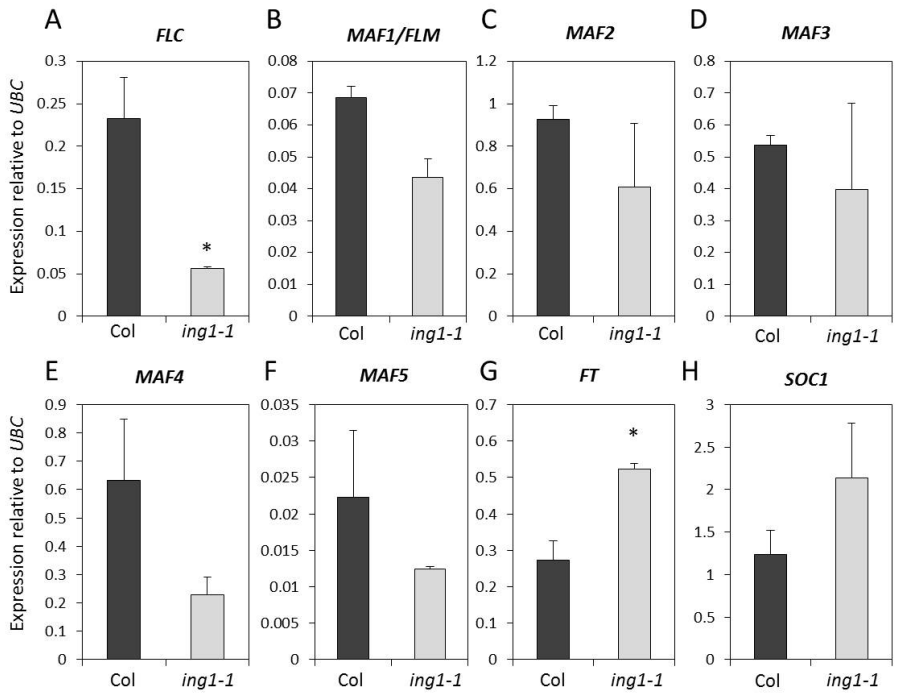

Figure 35. *ING1* is required for the regulation of master regulators of flowering. Expression of *FLC* **(A)**, *MAF1*/*FLM* **(B)**, *MAF2* **(C)**, *MAF4* **(D)**, *MAF5* **(E)**, *FT* **(F)** and *SOC1* **(G)** in Col and *ing1-1*, analyzed by qPCR in 10-day-old seedlings grown under LD conditions . *UBC* expression was used as control. *P < 0.05 with Student's *t*-test.

3.1.4. Arabidopsis ING2 *is required to activate the floral transition*

To characterize the function of *ING2*, we searched for insertion mutants for this gene. We identified two lines, namely, GABI_166D07 and GABI_909H04, designated as *ing2-1* and *ing2-2*, respectively (Fig. 36A). The *ing2-1* line bears a T-DNA insertion in exon 5 and *ing2-2* in exon 3. We analyzed the expression of *ING2* in these mutants using primers upstream (*ING2 UPSTREAM*) and downstream (*ING2 DOWNSTREAM*) the *ing2- 1* insertion. When we used the *ING2 UPSTREAM* primer pair, the expression of *ING2* in *ing2-1* was not reduced, but slightly upregulated, while in *ing2-2*, it was reduced to approximately 35% of wt levels (Fig. 36B). Conversely, when we used the *ING2 DOWNSTREAM* primers, the expression of *ING2* was completely abolished in both mutant alleles (Fig. 36C). We also included the *ing1-1* mutant in this analysis, and using both primer combinations we observed that the expression of *ING2* is increased in this mutant allele. These results indicate that *ing2-1* and *ing2-2* are likely loss-of-function alleles, as they are unable to generate full length *ING2* proteins, and these mutations cause an increase in the expression of *ING1*. However, we cannot rule out the possibility that the incomplete transcripts that are produced in both alleles could generate a truncated version of the protein, which would be highly expressed in *ing2-1* mutants.

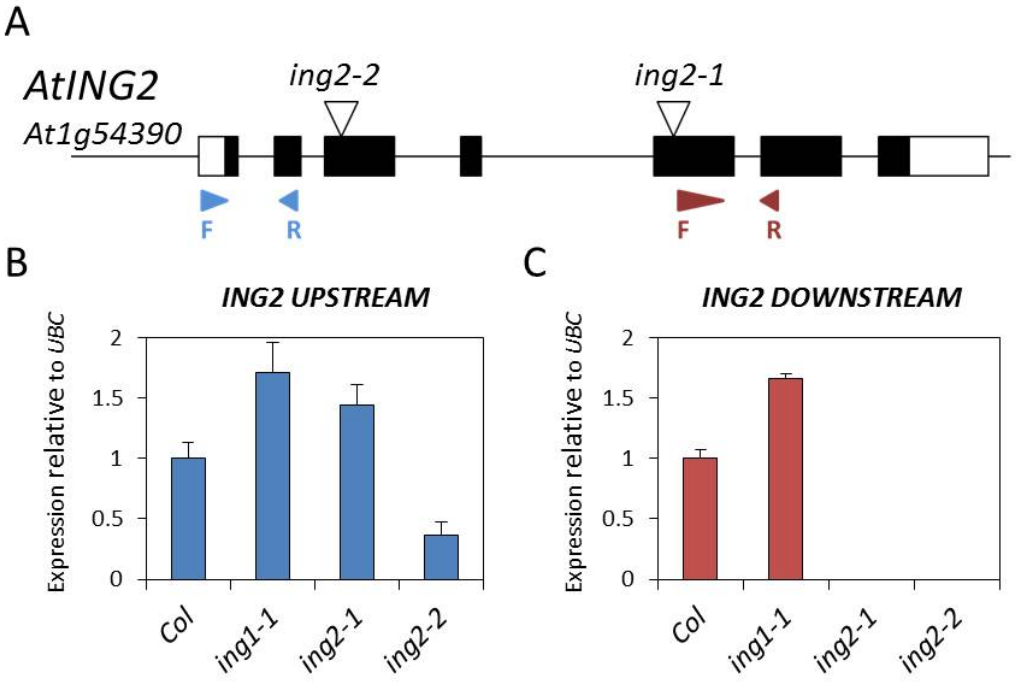

Figure 36. The *ING2* gene in *Arabidopsis thaliana* and insertion alleles isolated. (A) Gene structure of *AtING2* and T-DNA insertion lines. Boxes represent exons and the black line represents the introns. The white boxes represent the 5' and 3' UTRs (left and right, respectively). Inverted triangles represent the position of the T-DNA insertions in the *ing2-1* and *ing2-2* alleles. Arrows represent the position of the forward (F) and reverse (R) primers used to determine the expression of *ING2* upstream (blue) and downstream (red) the *ing2-1* T-DNA insertion. Expression of *ING2* in Col, *ing1-1*, *ing2-1* and *ing2-2,* analyzed by qPCR using primers upstream **(B)** and downstream **(C)** the T-DNA insertion in *ing2-1.* In **(B)** and **(C)**, expression of *ING2* was analyzed in 10-day-old seedlings grown under LD conditions. *UBC* expression was used as a control.

We examined the flowering time phenotype of *ing2-1* and *ing2-2* plants in LD and SD conditions in order to investigate their possible role in the regulation of the floral transition. Under LD, both *ing2-1* and *ing2-2* flowered later than the wt, and this delay in flowering was more noticeable in the case of *ing2-2* (Fig. 37A, B). Under SD *ing2-1* plants also showed a late flowering phenotype. Under these conditions, *ing2-2* plants showed more extreme phenotypic alterations and were unable to flower, producing on average more than 135 leaves before showing symptoms of senescence and dying (Fig. 37C, D). These results indicate that *ING2* plays an opposite role to *ING1* in the regulation of flowering, as it is required to promote the floral transition in LD and SD. In addition, the *ing2-2* seems to be a stronger allele than *ing2-1* regarding flowering time, and this might be related to the location of the T-DNA in the mutant lines, which is closer to the initiation codon in the *ing2*-2 allele (Fig. 36A).

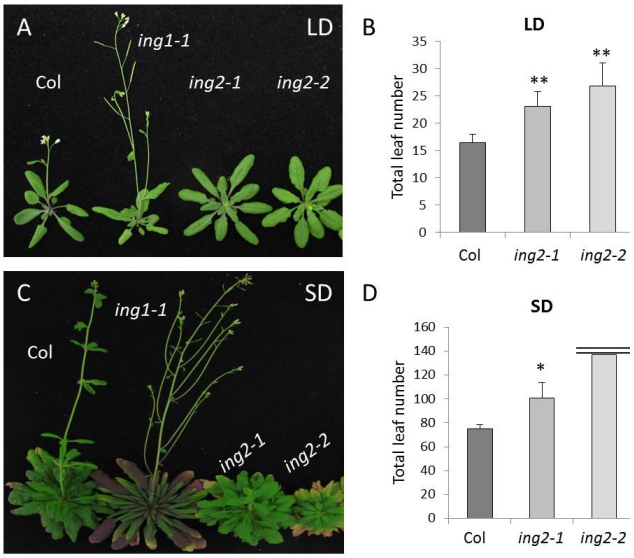

Figure 37. Mutations in *ING2* cause late flowering both under LD and SD. (A) Flowering time phenotype of Col, *ing1-1*, *ing2-1* and *ing2-2* plants grown in LD. **(B)** Total leaf number at flowering of Col, *ing1-1*, *ing2-1* and *ing2-2* plants in LD. **(C)** Flowering time phenotype Col, *ing1-1*, *ing2-1* and *ing2-2* plants grown in SD. **(D)** Total leaf number at flowering of Col, *ing1-1*, *ing2-1* and *ing2-2* plants in SD. The two parallel lines on top of the bar corresponding to *ing2-2* represent the number of leaves produced before senescence and death of the plants without flowering. *P < 0.05 with Student's t-test. **P < 0.01 with Student's t-test.

3.1.5. ING2 *is required for proper flower development*

Apart from the delay in flowering observed in the *ing2* mutants, we also identified some abnormalities in the flower development of these mutants. The flowers of the *ing2-2* mutants showed an unusual number of petals, with some flowers having extra petals and a disrupted floral architecture, and some others showing just as few as two (Fig. 38A). Also, this phenotype seemed to be exclusive for *ing2* mutants, as *ing1-1* flowers showed no alterations. Among the plethora of genes that control the onset of flower formation and flower development, *FUL* and *PI* play important roles in floral meristem determination and floral organ specification (Goto and Meyerowitz, 1994; Wuest et al., 2012; Ferrandiz et al., 2000; Yamaguchi et al., 2009). We found these genes to be upregulated in *ing2-2* seedlings (Fig. 38B, C), which might explain the phenotypic alterations observed in these mutants.

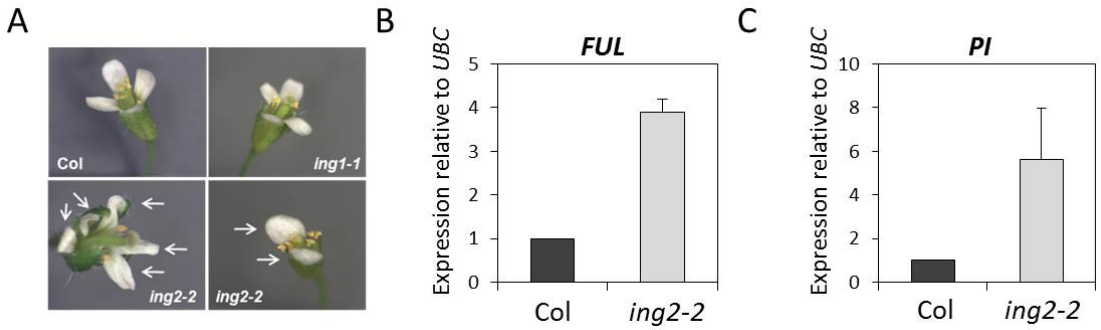

Figure 38. *ING2* is required for proper flower development. (A) Flowers of Col, *ing1-1* and *ing2-2* plants. Petals in *ing2-2* flowers showing abnormal number and distribution are marked with white arrows. Expression of *FUL* **(B)** and *PI* **(C)** analyzed by qPCR in 10-day-old seedlings of Col and *ing2-2* grown in LD. *UBC* expression was used as a control. Values in Col were set to 1.

3.1.6. *A* 35S::MYC-ING2 *construct complements the phenotypic alterations of* ing2-2 *plants*

To confirm that the phenotypic alterations observed in *ing2-2* mutants were a consequence of the *ing2-2* mutation, we performed a complementation experiment with the wt version of *ING2*. We generated a *35S::Myc-ING2* construct and we transformed it into an *ing2-2* background, and several independent transgenic lines were isolated and analyzed for phenotypic alterations. In these lines, both the flowering phenotype and the flower development phenotype were restored (Fig. 39A, B, C). These results indicate that the MYC-ING2 fusion protein introduced is functional and complements the *ing2-2* phenotype, and confirms that the phenotypic alterations observed in this mutant are caused by the mutation in *ING2*. We corroborated that these lines overexpress *ING2* by qPCR (Fig. 39D), reaching up to 4 times the expression levels of wt plants. We were also able to detect the Myc-ING2 fusion protein in seedling protein extracts using an anti-Myc antibody (Fig. 39E).

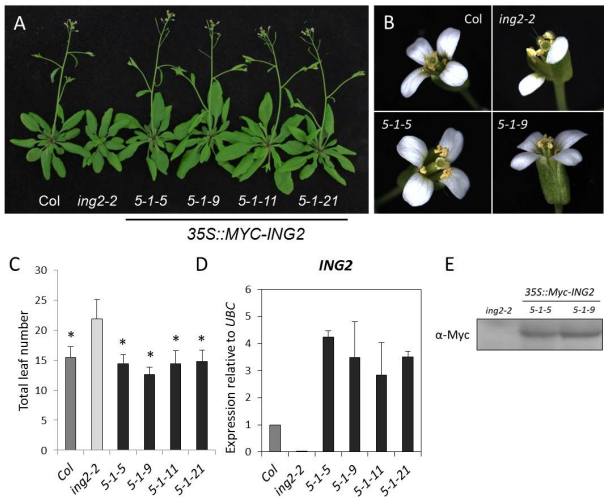

Figure 39. A *35S::Myc-ING2* construct complements the flowering time and flower development defects of *ing2-2* mutant. (A) Flowering time phenotype of Col, *ing2-2*, and the *35S::Myc-ING2* lines *5-1-5*, *5-1-9*, *5-1-11* and *5-1-21* grown under LD. **(B)** Flowers of Col, *ing2-2*, and the *35S::MycING2* lines *5-1-5* and *5-1-9.* **(C)** Total leaf number at flowering of Col, *ing2-2*, and the *35S::Myc-ING2* lines *5-1-5*, *5-1-9*, *5-1-11* and *5-1-21* grown under LD. **(C)** Expression of *ING2* in Col, *ing2-2*, and the *35S::Myc-ING2* lines *5-1-5*, *5-1-9*, *5-1-11* and *5-1-21* analyzed by qPCR in 10-day-old seedlings grown under LD conditions. *UBC* expression was used as a control. **(E)** Western blot detection of Myc-ING2 with an α-Myc antibody in *35S::Myc-ING2* lines *5-1-5* and *5-1-9. ing2-2* plants were used as negative controls. *P < 0.01 with Student's *t*-test when compared with *ing2-2*.

3.1.7. ING2 *is required for the control of master regulators of flowering*

To determine whether the late flowering phenotype observed in *ing2-2* plants correlated with alterations in the expression of master floral regulators, we measured the expression of *FLC*, *MAF1*/*FLM*, *MAF2*, *MAF4*, *MAF5*, *FT* and *SOC1* in Col and *ing2-2* plants by qPCR (Fig. 40). We found no significant alterations in the expression of *FLC* and *MAF5*, and only slight increases in the expression of *MAF1*/*FLM*, *MAF2* and *MAF4*. Conversely, we observed that the floral integrators *SOC1*, and especially *FT* were downregulated. This strongly reduced

expression of *FT* is in agreement with the late flowering phenotype of *ing2-2*. These results indicate that *ING2* is involved in the activation of flowering by regulating the floral integrators *FT* and *SOC1*.

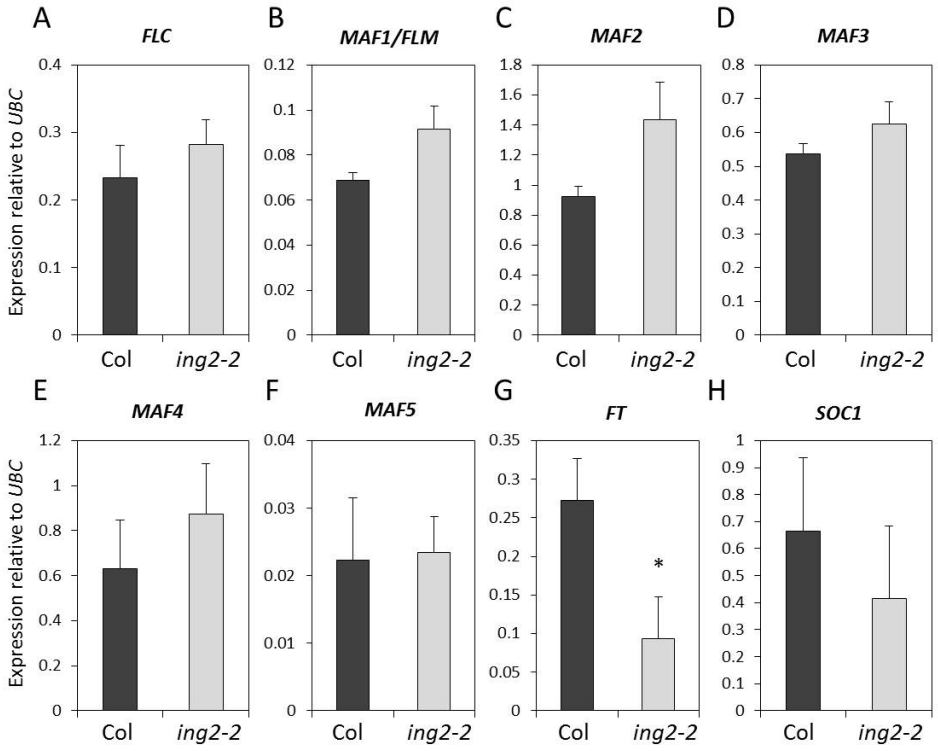

Figure 40. *ING2* is required for the regulation of master regulators of flowering. Expression of *FLC* **(A)**, *MAF1*/*FLM* **(B)**, *MAF2* **(C)**, *MAF4* **(D)**, *MAF5* **(E)**, *FT* **(F)** and *SOC1* **(G)** in Col and *ing2-2*, analyzed by qPCR in 10-day-old seedlings grown in LD conditions. *UBC* expression was used as a control. *P < 0.05 with Student's *t*-test.

3.1.8. ING2 *is epistatic to* ING1 *in the regulation of flowering*

To investigate a possible functional relationship between *ING1* and *ING2* in the control of flowering time, we crossed *ing1-1* with *ing2-2* and generated *ing1-1 ing2-2* double mutant plants. We analyzed its flowering time under LD and observed that these plants displayed a late flowering phenotype, very similar to that of *ing2-2* plants (Fig. 41A, B). We also measured flowering time in this double mutant under SD and, similarly to *ing2-2* single mutant, *ing1-1 ing2-2* double mutant plants were unable to flower under non-inductive photoperiods (Fig. 41C, D). In this case, the double mutant plants produced more than 200 leaves on average, highlighting that in these plants, the floral transition is severely compromised. These results indicate that *ING2* is epistatic to *ING1* in the regulation of flowering under both photoperiods.

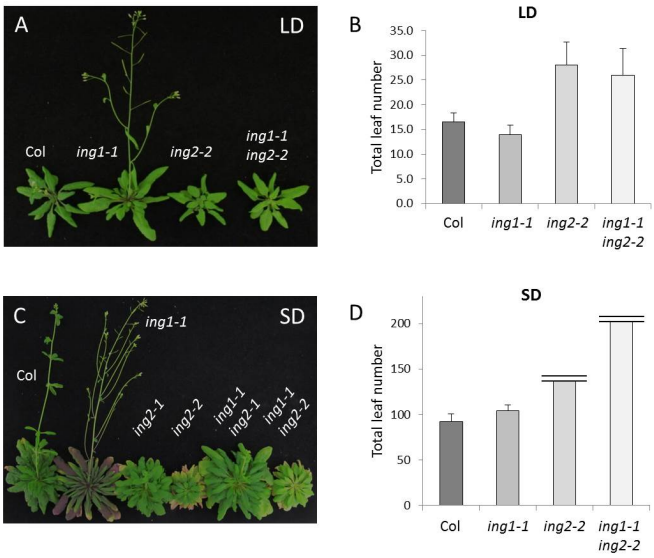

Figure 41. *ING2* is epistatic to *ING1* in the regulation of flowering time under LD and SD. (A) Flowering time phenotype of Col, *ing1-1*, *ing2-2* and *ing1-1 ing2-2* plants grown in LD. **(B)** Total leaf number at flowering of Col, *ing1-1*, *ing2-2* and *ing1-1 ing2-2* plants in LD. **(C)** Flowering time phenotype Col, *ing1-1*, *ing2-1*, *ing2-2*, *ing1-1 ing2-1* and *ing1-1 ing2-2* plants grown in SD. **(D)** Total leaf number at flowering of Col, *ing1-1*, *ing2-1* and *ing2-2* plants in SD.

3.1.9. ING2 *is epistatic to* ING1 *in the regulation of* FLC *and* FT *in LD*

We asked if the late flowering phenotype observed in *ing1-1 ing2-2* in LD was due to changes in the expression of key genes controlling the floral transition. To answer this, we measured the expression of the floral repressor *FLC* and the floral integrator *FT* in *ing1-1 ing2-2* plants grown under LD. *ing1-1* and *ing2-2* single mutants were also included in this analysis. We observed that, consistently with previous results, the expression of *FLC* was reduced in *ing1-1*, and remained unchanged in *ing2-2*. The expression of this gene in the *ing1-1 ing2-2* double mutant was very similar to that *ing2-2* (Fig. 42A). The expression of *FT* was increased in *ing1-1* and decreased in *ing2-2*, also consistently with our previous observations. The expression of *FT* observed in *ing1-1 ing2-2* was again comparable with the one observed in *ing2-2* (Fig. 42B). The similar expression levels of *FLC* and *FT* in *ing2-2* and *ing1-1 ing2-2* plants are in agreement with the late flowering phenotype observed in these plants and indicate that *ING2* is epistatic to *ING1* also in the regulation of *FLC* and *FT* under LD photoperiods.

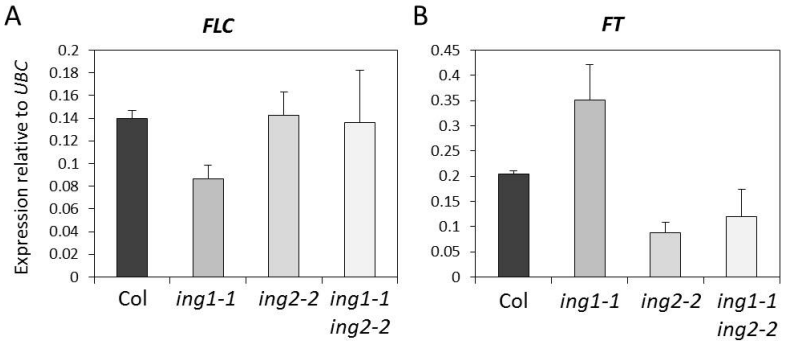

Figure 42. *ING2* is epistatic to *ING1* in the regulation of *FLC* and *FT* expression under LD. Expression of *FLC* **(A)** and *FT* **(B)** in Col, *ing1-1*, *ing2-2* and *ing1-1 ing2-2*, analyzed by qPCR in 10-day-old seedlings grown under LD conditions. *UBC* expression was used as a control.

3.1.10. The ing1-1 ing2-2 *double mutant shows pleiotropic phenotypic alterations*

We decided to study in more detail some phenotypic traits of *ing1-1 ing2-2* plants. We noticed that, under SD conditions, the rosette of *ing2-2* and *ing1-1 ing2-2* plants was conspicuously smaller. When we measured the rosette diameter in these two genotypes we observed that it was reduced in approximately 40% compared to Col and *ing1-1* (Fig. 43A). The siliques of *ing1-1 ing2-2* were also notably smaller compared to Col and the single mutants (Fig. 43C, D). In addition, we found that a portion of the seeds in the *ing1-1 ing2-2* siliques were aborted (Fig. 43B), suggesting that in the absence of both *ING1* and *ING2*, gametophyte development might be impaired.

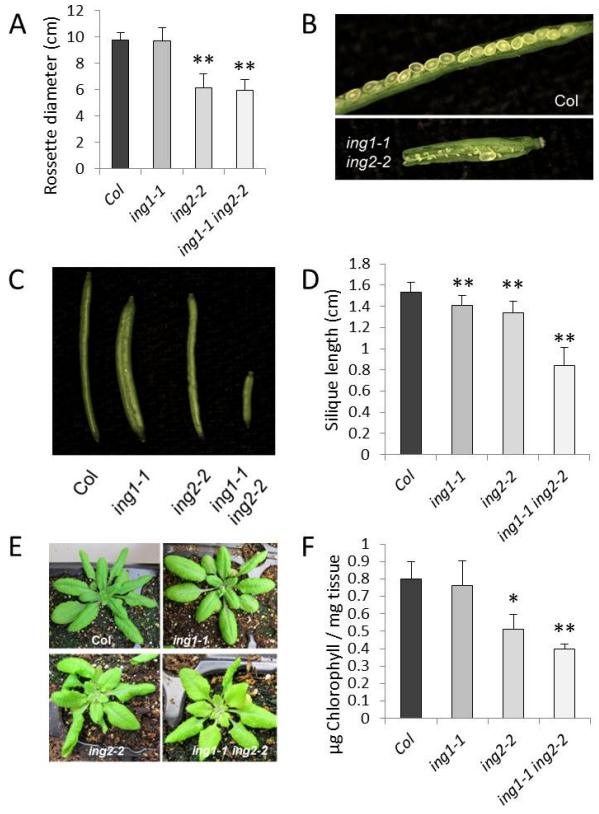

Figure 43. The *ing1-1 ing2-2* double mutant displays pleiotropic phenotypic alterations. (A) Rossette diameter (cm) of Col, *ing1-1*, *ing2-2* and *ing1-1 ing2-2* plants grown in SD. **(B)** Section of siliques of Col and *ing1-1 ing2-2*. Aborted seeds are present in *ing1-1 ing2-2* siliques. **(C)** Phenotype of Col, *ing1-1*, *ing2-2* and *ing1-1 ing2-2* siliques. **(D)** Quantification of silique length (cm) in Col, *ing1-1*, *ing2-2* and *ing1-1 ing2-2* plants grown in LD. **(E)** Phenotype of Col, *ing1-1*, *ing2-2* and *ing1-1 ing2-2* plants grown in LD. Symptoms of chlorosis can be seen in *ing2-2* and *ing1-1 ing2-2* plants. **(F)** Quantification of chlorophyll concentration as ug of chlorophyll per mg of tissue in 10-day-old Col, *ing1-1*, *ing2-2* and *ing1-1 ing2-2* seedlings grown in LD. * $P < 0.05$ and ** $P < 0.01$ with Student's *t*-test.

We also noticed evident symptoms of chlorosis in *ing2-2* and *ing1-1 ing2-2* plants, as they looked paler than Col and *ing1-1* plants (Fig. 43E). In fact, the chlorophyll concentration of these plants is reduced compared to the wt, and is lower in *ing1-1 ing2-2* than in *ing2-2* (Fig. 43F).

These results indicate that ING2 has an important role in the control of some phenotypic traits such as rosette diameter, with no apparent contribution of ING1. However, *ING1* and *ING2* play redundant roles in the control of the silique development, chlorophyll synthesis and gametophyte development.

3.1.11. Warm growing temperatures suppress the late flowering phenotype of ing2-2 and ing1-1 ing2-2

As mentioned above in the introduction section, the role of ambient temperature on flowering is starting to be elucidated (Capovilla et al., 2015). Warm growing temperatures of 27-28ºC accelerate flowering in Arabidopsis and cause an upregulation of *FT* that could mediate this alteration of flowering time (Pose et al., 2013). Plants with mutations in *ARP6* have a similar phenotype and transcriptomic profiles to that of wt plants grown in warm temperatures (Kumar and Wigge, 2010), and H2A.Z mediates the thermosensory response in Arabidopsis (Jarillo and Pineiro, 2015). Given the functional relationship described for the NuA4 and the SWR1 (Lu et al., 2009; Altaf et al., 2010; Auger et al., 2008), we decided to assess the effect of warm growing temperatures on flowering in *ing* mutants. For that, we carried out a flowering experiment in which we grew Col, *ing1-1*, *ing2-2* and *ing1-1 ing2-2* plants under LD at 22ºC and 27ºC (Fig. 44). In this assay we included the *esd1-10* mutant as a control. As expected, warm temperatures accelerated flowering in Col plants, and the flowering phenotype of *esd1-10* was very similar at both growing temperatures. The flowering phenotype of *ing1-1* was earlier than Col in both photoperiodic conditions. However, we observed that warm temperatures suppressed the late flowering phenotype of *ing2-2* and *ing1-1 ing2-2*, and at 27ºC they flowered with the same number of leaves as Col plants (Fig. 44). As we have mentioned, under standard growing conditions, these mutants show reduced levels of *FT*. It is possible that in the absence of *ING2*, the upregulation of *FT* in warm temperatures is sufficient to restore flowering time to wt comparable levels.

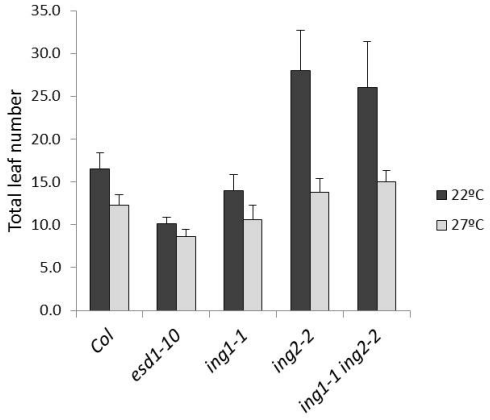

Figure 44. Warm ambient temperatures suppress the late flowering phenotype of *ing2-2* and *ing1-1 ing2-2*. Total leaf number at flowering of Col, *esd1-10*, *ing1-1*, *ing2-2* and *ing1-1 ing2-2* plants grown in LD at 22ºC (dark gray) and 27ºC (light gray).

3.2. Genetic analysis of AtING1 *and* AtING2

3.2.1. ING1 *and* ING2 *genetically interact with* FLC *in the regulation of flowering time*

Members of the Arabidopsis SWR1 complex such as ARP6 and SWC6 have been shown to control flowering time through the regulation of the floral repressor *FLC* (Martin-Trillo et al., 2006; Lazaro et al., 2008). Also, unpublished results in our lab suggest that the Arabidopsis homologues of some subunits shared between yeast SWR1 and NuA4 (SWC4, YAF9) might also be involved in the regulation of *FLC*. We decided to perform genetic analyses to test if the

flowering time alterations observed in *ing1-1* and *ing2-2* depend on *FLC*. For that we crossed *ing1-1* with the *FLC* null allele *flc-3* (Michaels and Amasino, 1999) and generated *ing1-1 flc-3* double mutant plants. Quantification of the flowering time phenotype showed that these plants flowered at the same time and with the same number of leaves as the parental single mutants *ing1-1* and *flc-3*, indicating that in the absence of *FLC*, mutations in *ING1* do not further accelerate flowering (Fig. 45A, B). We also checked the expression of *FT*, the main output of *FLC* function, and concluded that *ing1-1 flc-3* plants showed an up-regulation of *FT* comparable to that observed in the *ing1-1* mutant (Fig. 45C). These results indicate that *ING1* and *FLC* regulate flowering through the same genetic pathway. Also, the expression of *FT* observed in *ing1-1 flc-3* indicates that the lack of *FLC* in an *ing1-1* background does not induce further up-regulation of *FT* and suggests that the effect of *ING1* in flowering is mainly due to its regulation over *FLC*.

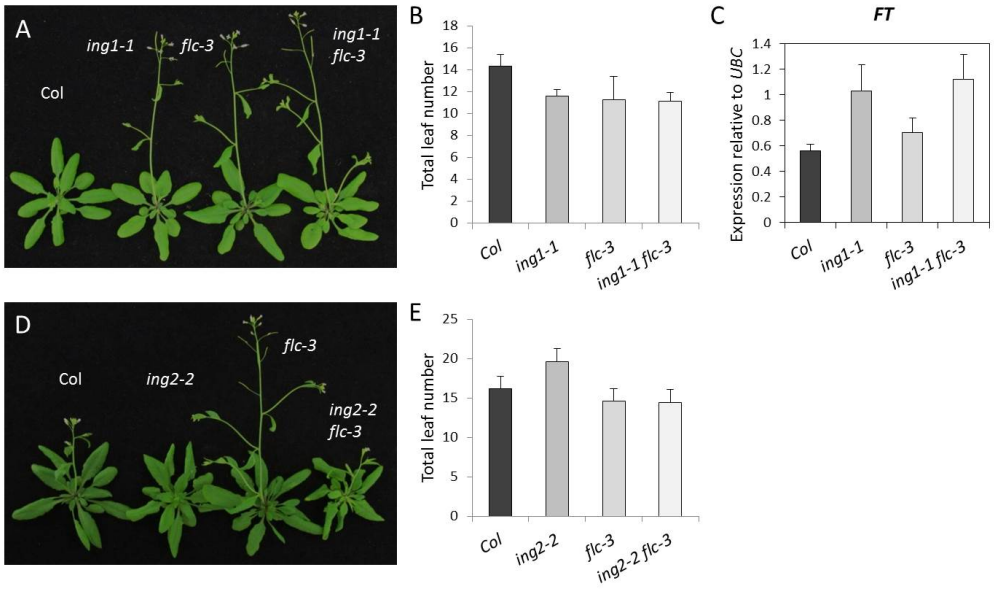

Figure 45. *ING1* and *ING2* genetically interact with *FLC*. Flowering time phenotype **(A)** and total leaf number at flowering **(B)** of Col, *ing1-1*, *flc-3* and *ing1-1 flc-3* plants grown in LD. **(C)** Expression of *FT* in Col, *ing1-1*, *flc-3* and *ing1-1 flc-3*, analyzed by qPCR in 10-day-old seedlings grown in LD conditions. *UBC* expression was used as a control. Flowering time phenotype **(D)** and total leaf number at flowering **(E)** of Col, *ing2-2*, *flc-3* and *ing2-2 flc-3* plants grown in LD.

We also crossed the *ing2-2* mutant with *flc-3* and analyzed the flowering of the *ing2-2 flc-3* double mutant. In this case, we observed that the *flc-3* mutation completely suppresses the late flowering phenotype of *ing2-2*, indicating that *ING2* requires an active *FLC* gene to activate the floral transition (Fig. 45D, E).

3.2.2. Mutations in ING1 *and* ING2 *cause alterations in flowering time in a* FRI *background.*

To get a deeper insight in the regulation of *ING1* and *ING2* over *FLC*, we introduced the *ing1-1*, *ing2-1* and *ing2-2* mutations into a *FRI Sf2* background, which has an active allele of *FRI*, high levels of *FLC* and a very late flowering phenotype (Michaels and Amasino, 1999). We

analyzed the flowering time in these plants and monitored *FLC* expression levels at three time points: day 8, 10, and 12 after germination. *arp6-1 FRI* plants were also included as a control. This analysis revealed that the *ing1-1* mutation partially suppresses the late flowering phenotype of Col *FRI* plants (Fig. 46A, B), as *ing1-1 FRI* plants flowered earlier than Col *FRI* plants. This acceleration of flowering correlated with lower levels of *FLC* at the three time points analyzed (Fig. 46C).

When we introduced the *ing2-1* and *ing2-2* mutations in a *FRI* background we observed that these plants flowered much later than the parental Col *FRI*, and this delay was more prominent in the case of *ing2-2 FRI* (Fig. 46A, B). In addition, *FLC* was upregulated in *ing2-1 FRI* and *ing2-2 FRI* plants compared to Col *FRI*, especially in *ing2-2 FRI* plants (Fig. 46C). As expected, *arp6-1 FRI* plants display very low levels of *FLC* expression (Fig. 46C).

These results indicate that the *ing1-1* mutation also accelerates flowering and downregulates *FLC* expression in a background with high *FLC* levels. In contrast, mutations in *ING2* have the opposite effect and delay flowering in a *FRI* background, and this delay correlates with increased *FLC* expression.

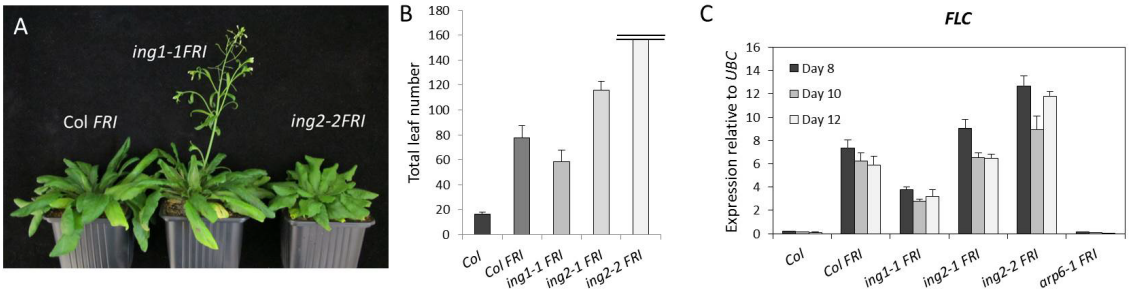

Figure 46. *ing1-1* and *ing2-2* mutants show opposite phenotypes and *FLC* expression in *FRI* background. (A) Flowering time phenotype of Col *FRI*, *ing1-1 FRI* and *ing2-2 FRI* plants grown in LD. **(B)** Total leaf number at flowering of Col, Col *FRI*, *ing1-1 FRI, ing2-1 FRI* and *ing2-2 FRI* plants in LD. **(C)** Expression of *FLC* in Col, Col *FRI*, *ing1-1 FRI*, *ing2-1 FRI*, *ing2-2 FRI* and *arp6-1 FRI*, analyzed by qPCR in 10-day-old seedlings grown under LD conditions. *UBC* expression was used as a control.

3.2.3. ING1 *acts additively with SWR1 components to regulate flowering time*

Our genetic and expression data indicate that *ING1* might regulate flowering time by regulating *FLC*. As mentioned above, SWR1 components also regulate *FLC* expression and a functional interplay between SWR1 and NuA4 complexes has been described in yeast (Lu et al., 2009; Altaf et al., 2010; Auger et al., 2008). To evaluate the possible cooperation between Arabidopsis SWR1 and ING1 in the control of flowering time, we combined the *ing1-1* mutation with mutations in components of Arabidopsis SWR1 like *esd1-10* (*ARP6* allele) and *swc6-1* (Martin-Trillo et al., 2006; Lazaro et al., 2008). *ING1* acts additively with *ESD1/ARP6* and *SWC6* in the regulation of flowering time in LD, as *ing1-1 esd1-10* and *ing1-1 swc6-1* plants flower earlier than their respective parental single mutants (Fig. 47A, B, D, E). This additive phenotype can also be seen in the transcriptional regulation of *FLC*, since the levels of *FLC* in *ing1-1 esd1-10* and *ing1-1 swc6-1* plants are much lower than in the parental single mutants, to the point that they are barely detectable (Fig. 47C, F).

These results indicate that *ING1* regulates flowering time and activates *FLC* expression independently of *ARP6* and *SWC6*, and suggests functional independence between this putative Arabidopsis NuA4 subunit and SWR1.

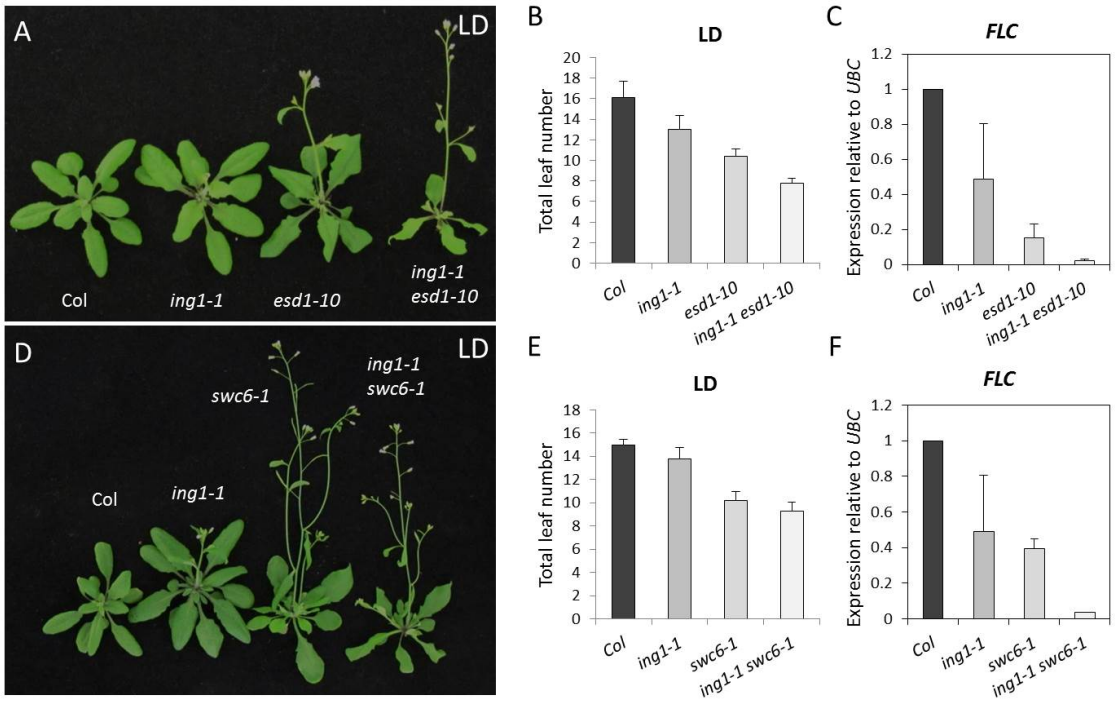

Figure 47. *ING1* acts additively with *ARP6* and *SWC6* to regulate flowering time in LD. Flowering time phenotype **(A)** and total leaf number at flowering **(B)** of Col, *ing1-1*, *esd1-10* and *ing1-1 esd1-10* plants grown in LD. **(C)** Expression of *FLC* in Col, *ing1-1*, *esd1-10* and *ing1-1 esd1-10*. Flowering time phenotype **(D)** and total leaf number at flowering **(E)** of Col, *ing1-1*, *swc6-1* and *ing1-1 swc6-1* plants grown in LD. **(F)** Expression of *FLC* in Col, *ing1-1*, *swc6-1* and *ing1-1 swc6-1*. In **(C)** and **(F)**, expression of *FLC* was analyzed by qPCR in 10-day-old seedlings grown in LD. *UBC* expression was used as a control.

3.2.4. Mutations in SWR1 components suppress the late flowering phenotype of ing2-2 *mutant plants*

To unveil possible genetic interactions of *ING2* with members of the SWR1 complex, we also combined the *esd1-10* and *swc6-1* mutations with *ing2-2*, and generated the corresponding double mutant plants. The analysis of these plants revealed that both *esd1-10* and *swc6-1* mutations suppress the late flowering phenotype of *ing2-2* under LD conditions (Fig. 48A, B; Fig. 49A, B). Under SD, the phenotype of *ing2-2* was also completely suppressed by *esd1-10* and *swc6-1* (Fig. 48D, E; Fig. 49D, E). In these conditions, *ing2-2 esd1-10* and *ing2-2 swc6-1* plants recovered the ability to flower and showed an additional acceleration of flowering, indicating that the activity of SWR1-C is required for the late flowering phenotype of *ing2-2*.

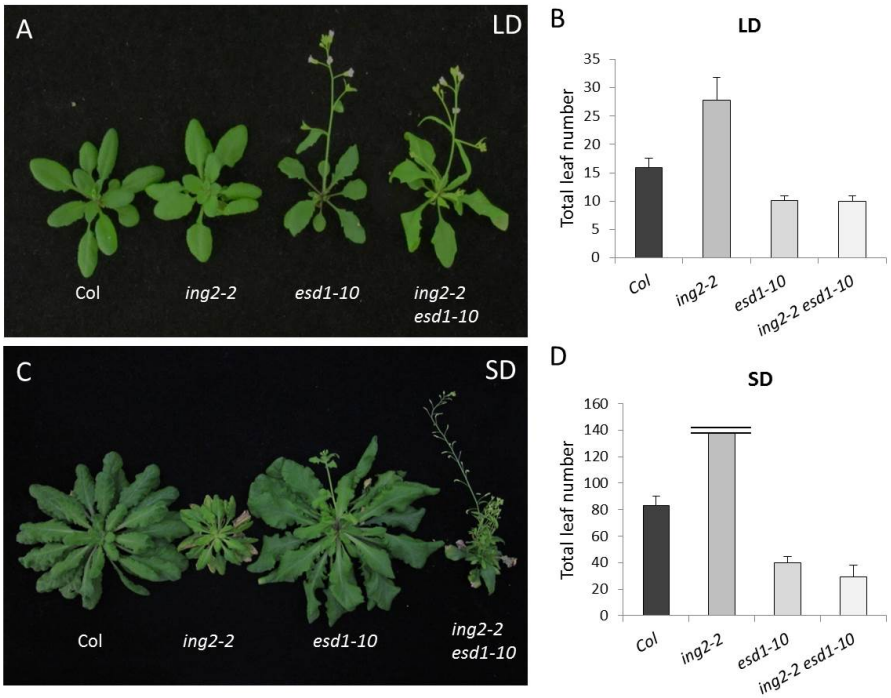

Figure 48. Mutations in *ARP6* suppress the late flowering phenotype of *ing2-2*. Flowering time phenotype **(A)** and total leaf number at flowering **(B)** of Col, *ing2-2*, *esd1-10* and *ing2-2 esd1-10* plants grown in LD. Flowering time phenotype **(C)** and total leaf number at flowering **(D)** of Col, *ing2-2*, *esd1-10* and *ing2-2 esd1-10* plants grown in SD.

The expression of *FLC* was examined in *ing2-2 swc6-1* plants grown under LD (Fig. 49C). These plants showed reduced levels of *FLC* compared to the *sw6-1* single mutant.

These genetic data and the reduced expression of *FLC* in *ing2-2 swc6-1* suggest that the late flowering phenotype of *ing2-2* depends on full activation of *FLC* by SWR1, and are consistent with our previous genetic data that indicate that mutations in *FLC* also suppress the late flowering phenotype of *ing2-2*.

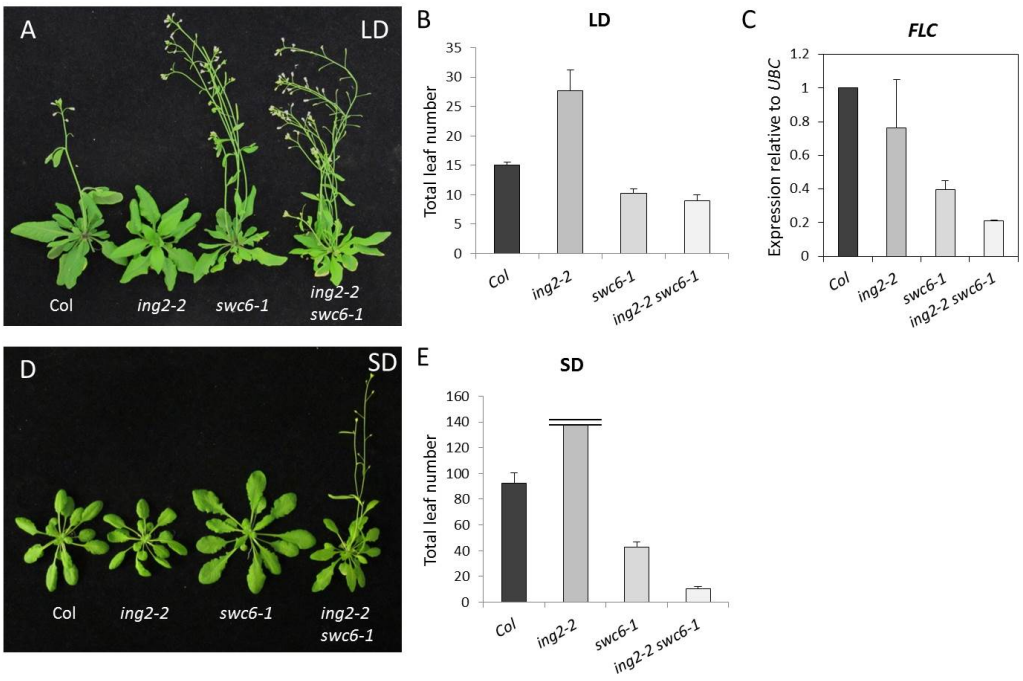

Figure 49. Mutations in *SWC6* suppress the late flowering phenotype of *ing2-2*. Flowering time phenotype **(A)** and total leaf number at flowering **(B)** of Col, *ing2-2*, *swc6-1* and *ing2-2 swc6-1* plants grown in LD. **(C)** Expression of *FLC* in Col, *ing2-2*, *swc6-1* and *ing2-2 swc6-1*, analyzed by qPCR in 10-day-old seedlings grown in LD conditions. *UBC* expression was used as a control. Flowering time phenotype **(D)** and total leaf number at flowering **(E)** of Col, *ing2-2*, *swc6-1* and *ing2-2 swc6-1* plants grown in SD.

3.2.5. Genetic relationship of ING1 *and* ING2 *with* ATX1 *and* ATXR7

The PHD domain present in ING1 and ING2 proteins has been shown to specifically bind H3K4me2/3 (Lee et al., 2009). The presence of this epigenetic mark is often associated with active transcription and several chromatin remodeling proteins have been demonstrated to modulate H3K4me3 levels at *FLC* (Crevillen and Dean, 2011). *ATX1* and *ATXR7* are required for the deposition of H3K4me3 at *FLC* and its transcriptional activation (Pien et al., 2008; Tamada et al., 2009; Berr et al., 2009), and therefore *atx1* and *atxr7* mutants show early flowering and reduced levels of *FLC*.

To find out if the outcome of *ING1* and *ING2* in flowering depends on H3K4me3 deposition mediated by ATX1 and ATXR7, we combined the *atx1-2* and *atxr7-2* mutations with mutations in *ING1* and *ING2*, and analyzed the flowering phenotype of these plants under LD conditions (Fig. 50A, B). Our results show that *ATX1* and *ATXR7* are both epistatic to *ING1*, as *ing1-1 atx1-2* and *ing1-1 atxr7-2* plants flower with the same number of leaves as the parental single mutants *atx1-2* and *atxr7-2*, suggesting that *ING1* regulates flowering through the same pathway as *ATX1* and *ATXR7*.

We also observed that double mutant combinations of *ing2-2* with *atx1-2* and *atxr7-2* flower much earlier than *ing2-2*, indicating that the *atx1-2* and *atxr7-2* mutations partially

suppress the late flowering phenotype of *ing2-2*, but not completely, revealing an additive function of these genes in the regulation of flowering time (Fig. 50C, D).

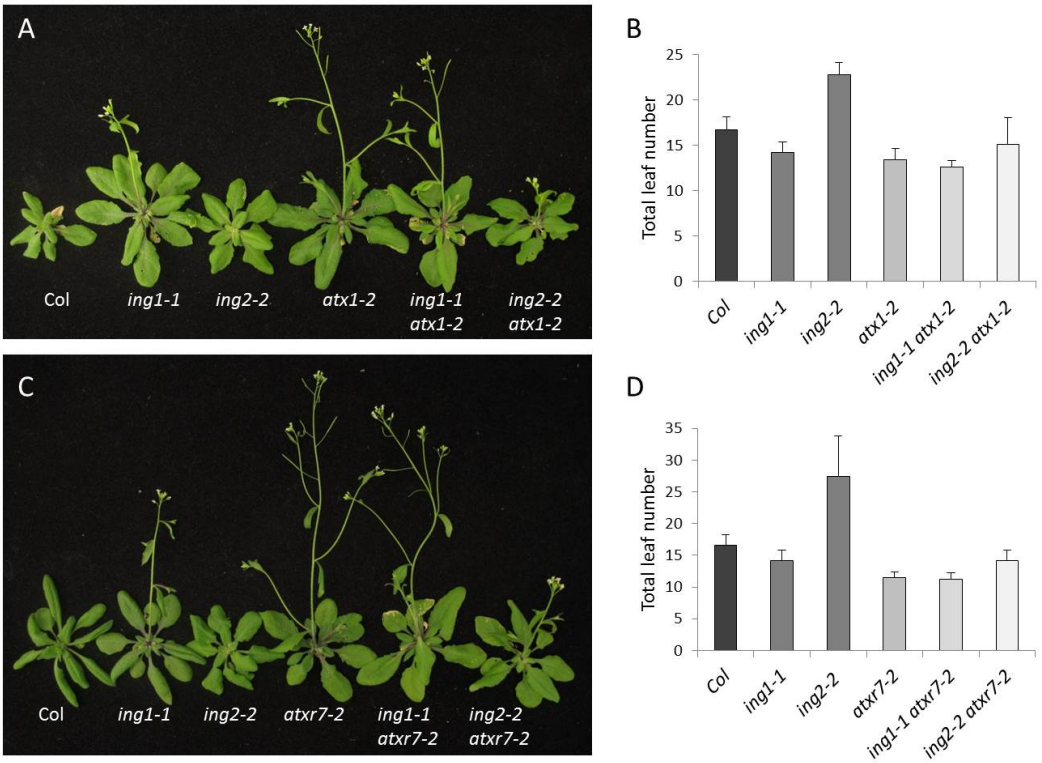

Figure 50. *ING1* but not *ING2* interacts genetically with *ATX1* and *ATRX7*. Flowering time phenotype **(A)** and total leaf number at flowering **(B)** of Col, *ing1-1*, *ing2-2, atx1-2, ing1-1 atx1-2 and ing2-2 atx1-2* plants grown in LD. Flowering time phenotype **(C)** and total leaf number at flowering **(D)** of Col, *ing1-1*, *ing2-2, atxr7-2, ing1-1 atxr7-2* and *ing2-2 atxr7-2* plants grown in LD.

3.2.6. ING1 *and* ING2 *genetically interact with* EBS*, a gene that encodes a reader protein of H3K4me3*

Our group has previously characterized the role of the PHD-containing protein EBS in the control of the floral transition. Previous studies showed that *EBS* is necessary for the repression of *FT* (Gomez-Mena et al., 2001; Pineiro et al., 2003), and recent observations revealed that EBS is involved in the chromatin-mediated repression of this floral integrator (Lopez-Gonzalez et al., 2014). EBS can bind genomic regions of *FT* and is required to maintain low levels of histone H3 acetylation in the chromatin of this locus. Moreover, the PHD domain present in EBS can bind H3K4me2/3, suggesting that the mechanism of repression by EBS involves the recognition of this epigenetic mark. Given that ING1 and ING2 have also been shown to recognize H3K4me2/3, we decided to investigate if there was a genetic interaction between *ING1* and *ING2* with *EBS*. For this purpose, we generated *ing1-1 ebs* and *ing2-2 ebs* double mutant plants and examined their flowering time under LD and SD.

We observed that under both LD and SD, the *ing1-1 ebs* double mutant flowered with the same number of leaves and was phenotypically very similar to the *ebs* single mutant, indicating that *EBS* is epistatic to *ING1* (Fig. 51). This result suggests that both proteins might regulate the floral transition through the same genetic pathway, although we cannot exclude the possibility that in a background with very high levels of *FT* such as *ebs*, mutations in *ING1* may not further accelerate flowering.

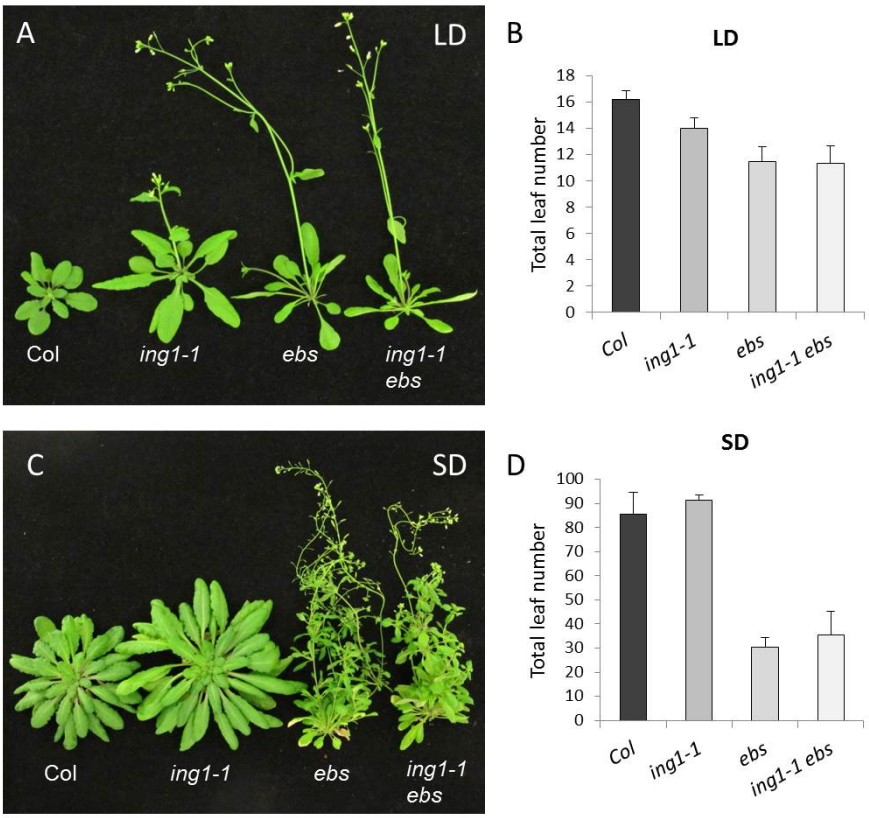

Figure 51. *EBS* is epistatic to *ING1* in the regulation of flowering time. **(A)** Flowering time phenotype of Col, *ing1-1*, *ebs* and *ing1-1 ebs* plants grown in LD. **(B)** Total leaf number at flowering of Col, *ing1-1*, *ebs* and *ing1-1 ebs* plants in LD. **(C)** Flowering time phenotype Col, *ing1-1*, *ebs* and *ing1-1 ebs* plants grown in SD. **(D)** Total leaf number at flowering of Col, *ing1-1*, *ebs* and *ing1-1 ebs* plants in SD.

Further analyses demonstrated that the *ing2-2 ebs* double mutant flowered with the same number of leaves as the *ebs* single mutant under both LD and SD (Fig. 52). This indicates that mutations in *EBS* suppress the late flowering phenotype of *ing2-2*. We also observed that, in this case, *ing2-2 ebs* plants were phenotypically different from the parental single mutants, especially under SD, showing dwarfism, terminal flowers, fasciated stems, and severe flower defects (not shown). These results indicate that *EBS* is required for the late flowering phenotype of *ing2-2*, and therefore *EBS* and *ING2* participate in the same genetic pathway to control flowering time, although they have independent roles in the control of other aspects of plant development. Alternatively, the high levels of *FT* expression in *ebs* might overcome the downregulation of this floral integrator observed in *ing2-2* and would explain the suppression of the late flowering phenotype observed in *ing2-2 ebs*.

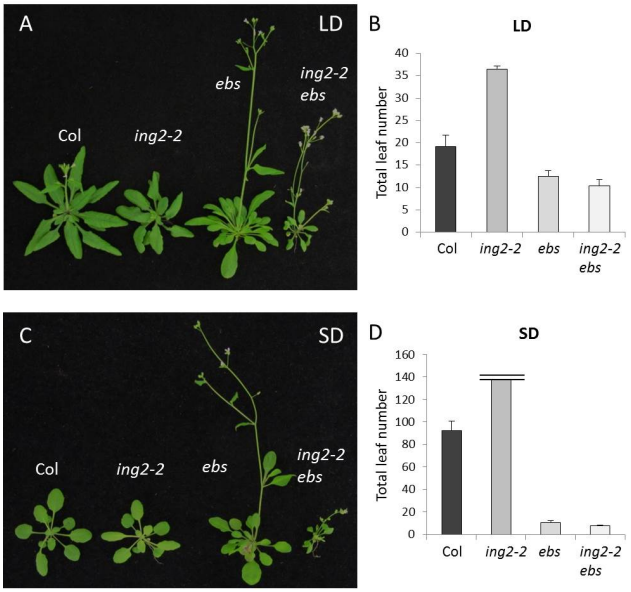

Figure 52. Mutations in *EBS* suppress the late flowering phenotype of *ing2-2*. (A) Flowering time phenotype of Col, *ing2-2*, *ebs* and *ing2-2 ebs* plants grown in LD. **(B)** Total leaf number at flowering of Col, *ing2-2*, *ebs* and *ing2-2 ebs* plants grown in LD. **(C)** Flowering time phenotype Col, *ing2-2*, *ebs* and *ing2-2 ebs* plants grown in SD. **(D)** Total leaf number at flowering of Col, *ing2-2*, *ebs* and *ing2-2 ebs* plants grown under SD.

3.2.7. Mutations in FT *delay flowering in* ing1-1 *and* ing2-2

To find out if the early flowering phenotype of *ing1-1* and the late flowering phenotype of *ing2-2* depend on *FT*, we crossed these mutants with *ft-10*, generated the corresponding double mutants and analyzed their flowering time under LD conditions (Fig. 53). In an *ft-10* background, the *ing1-1* mutation does not accelerate flowering, indicating that the early flowering phenotype of *ing1-1* requires an active *FT*. We also observed that *ing2-2 ft-10* plants flowered just slightly later than the parental *ft-10*. This indicates that the late flowering phenotype of *ing2-2* depends mainly on *FT*. The small delay in flowering observed in the *ing2-2 ft-10* double mutant may be due to an FT-independent role of ING2 in the regulation of flowering.

Figure 53. *ING1* and *ING2* interact genetically with *FT*. (A) Flowering time phenotype of Col, *ing1-1*, *ft-10* and *ing1-1 ft-10* plants grown in LD. **(B)** Flowering time phenotype of Col, *ing2-2*, *ft-10* and *ing2-2 ft-10* plants in LD. **(C)** Total leaf number at flowering of Col, *ing1-1*, *ing2-2*, *ft-10*, *ing1-1 ft-10* and *ing2-2 ft-10* plants in LD.

3.3. Analysis of protein interactions with other members of NuA4

3.3.1. ING1 and ING2 physically interact with core components of NuA4

Since AtING1 and AtING2 are homologues of yeast NuA4 components, these proteins might function in the context of a putative Arabidopsis NuA4 complex. For that reason we decided to test their physical interaction with other NuA4 subunits that we identified encoded in the Arabidopsis genome. Yeast Yng2 is part of the Piccolo NuA4 subcomplex, together with Epl1, Eaf6, and the catalytic subunit Esa1. Epl1 and Esa1 are essential for viability and unlike other subunits, they are only found in NuA4 (Allard et al., 1999; Clarke et al., 1999; Boudreault et al., 2003). Therefore, they can be considered core components of NuA4. Yeast Epl1 and Esa1 have been shown to physically interact (Boudreault et al., 2003; Chittuluru et al., 2011), and we investigated if that was also the case in Arabidopsis. For this reason, we analyzed the interaction between Arabidopsis HAM1 and EPL1 and between HAM1 and EPL2 proteins in Y2H assays. Yeast cells coexpressing HAM1 fused to the binding domain of GAL4 and EPL1 fused to the activation domain of GAL4 were able to grow on selective medium lacking histidine and supplemented with the histidine synthesis inhibitor 3-AT, indicating that these two proteins interact in yeast (Fig. 54A). The same result was obtained when the interaction between HAM1 and EPL2 was tested (Fig. 54B). This indicates that the NuA4 catalytic subunit HAM1 interacts with the Arabidopsis homologues of Epl1, supporting the existence of a Piccolo NuA4 subcomplex in Arabidopsis.

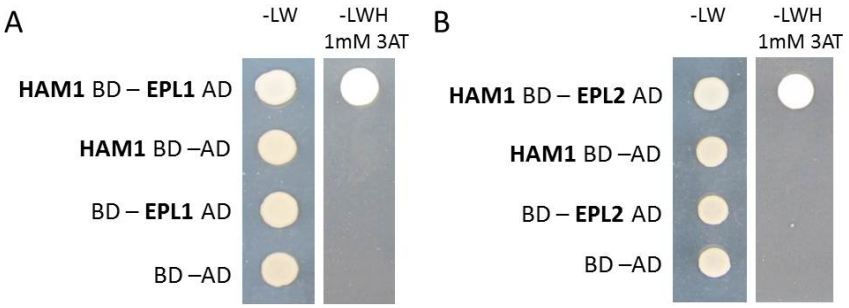

Figure 54. AtHAM1 physically interacts with AtEPL1 and AtEPL2 in Y2H assays. Interaction of HAM1 with EPL1 **(A)**, and HAM1 with EPL2 **(B)** tested in Y2H assay. HAM1 and EPL1/EPL2 full length proteins were fused to the DNA binding domain (BD) and activation domain (AD) of GAL4, respectively. Yeast cells carrying constructs and their combinations with the empty vectors were grown in non-selective medium (-LW) and selective medium (-LWH) supplemented with 1mM 3-amino-1.2.4-triazole (3AT).

Yeast Epl1 interacts with all the components of the Piccolo subcomplex and links it to the rest of the NuA4-C (Chittuluru et al., 2011). To assess whether Arabidopsis ING proteins can be part of a putative Piccolo NuA4 subcomplex, we tested by Y2H assays the interaction of ING1 and ING2 with EPL proteins and with EAF6. We found positive interactions between ING1 and EPL1 and ING2 and EPL2 (Fig. 55A, B). Furthermore, we observed that both ING1 and ING2 interact with EAF6 (Fig. 55C, D).

These Y2H results support the existence of a NuA4-like complex in Arabidopsis, and that ING1 and ING2 physically interact with members of this subcomplex, indicating that the

interactions between core components of the Piccolo NuA4 subcomplex are conserved in this model plant. These observations suggest that ING proteins might regulate gene expression as a part of Arabidopsis NuA4-C.

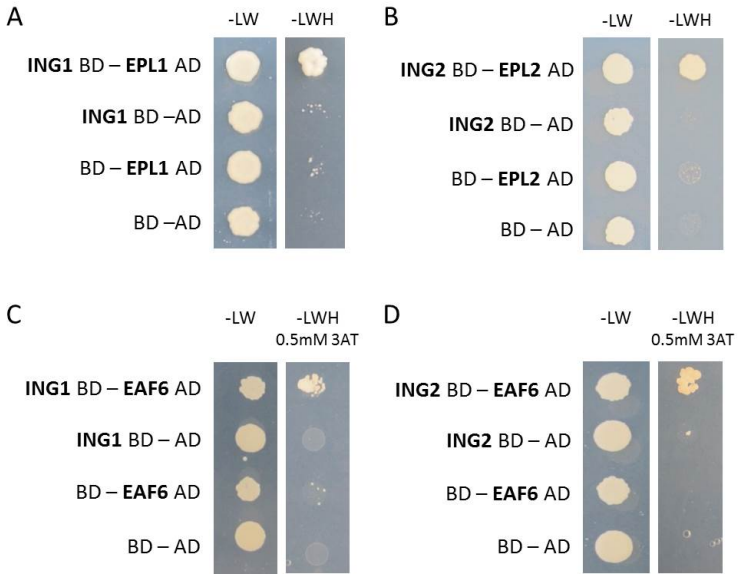

Figure 55. ING1 and ING2 physically interact with EPL proteins and EAF6. Interaction of ING1 with EPL1 **(A)** and EAF6 **(C)**, and ING2 with EPL2 **(B)** and EAF6 **(D)** tested in Y2H assays. ING1 and ING2 full length proteins were fused to the DNA binding domain (BD) GAL4, and EPL1, EPL2 and EAF6 full length proteins were fused to the activation domain (AD) of GAL4. Yeast cells carrying these constructs and their combinations with the empty vectors were grown in non-selective medium (-LW) and selective medium (-LWH) alone and supplemented with 0.5mM 3-amino-1.2.4-triazole (3AT).

3.4. Regulation of target genes of ING1 and ING2

3.4.1. ING1 and ING2 bind FLC chromatin

As shown above, our expression data indicate that ING1 is required for the regulation of *FLC*, while both *ING1* and *ING2* interact genetically with *FLC* in the control of flowering time. Moreover, the fact that ING1 and ING2 physically interact with other members of Piccolo NuA4 and the ability of their PHD domain to bind H3K4me3 (Lee et al., 2009) suggests that they might regulate gene expression by binding directly the chromatin of their target genes. To test if *FLC* is a direct target of ING1 and ING2, we used our *ING1* and *ING2* overexpression lines bearing tagged versions of ING proteins to perform ChIP experiments and analyze their binding to several chromatin regions in the *FLC* locus (Fig. 56A).

First, we analyzed the distribution of H3K4me3 along this locus, and we detected a strong peak in region *FLC3*, close to the TSS (Fig. 56B). This is consistent with previous reports (Tamada et al., 2009; Yang et al., 2014), and also with our results (Section 2.9). We then proceeded to analyze the binding of ING1 to *FLC*, using the *35S::FLAG-ING1* line 2-10-2, which complements the early flowering phenotype of *ing1-1* (Section 3.1.2). ChIP assays using an

anti-FLAG antibody allowed us to reveal an enrichment of immunoprecipitated DNA in our *35S::FLAG-ING1* line compared to Col in the region *FLC3*, which indicates that ING1 binds *FLC* chromatin, particularly to the region where H3K4me3 enrichment is highest (Fig. 56C).

Next, we also analyzed the possible binding of ING2 to *FLC* chromatin, using the *35S::Myc-ING2* lines 5-1-5 and 5-1-9, which complemented the late flowering phenotype and the floral phenotype of *ing2-2*. In this case, we detected a noticeable increase of immunoprecipitated DNA in regions *FLC3* and *FLC4* in both transgenic lines compared to Col, indicating that ING2 binds these two regions of *FLC* chromatin (Fig. 56D).

Altogether, these ChIP results indicate that *FLC* is a direct target of ING1 and ING2. Also, ING1 and ING2 bind *FLC* chromatin regions with higher H3K4me3 enrichment, supporting the idea that they regulate gene expression through the PHD-mediated recognition of this mark in the chromatin of their target genes.

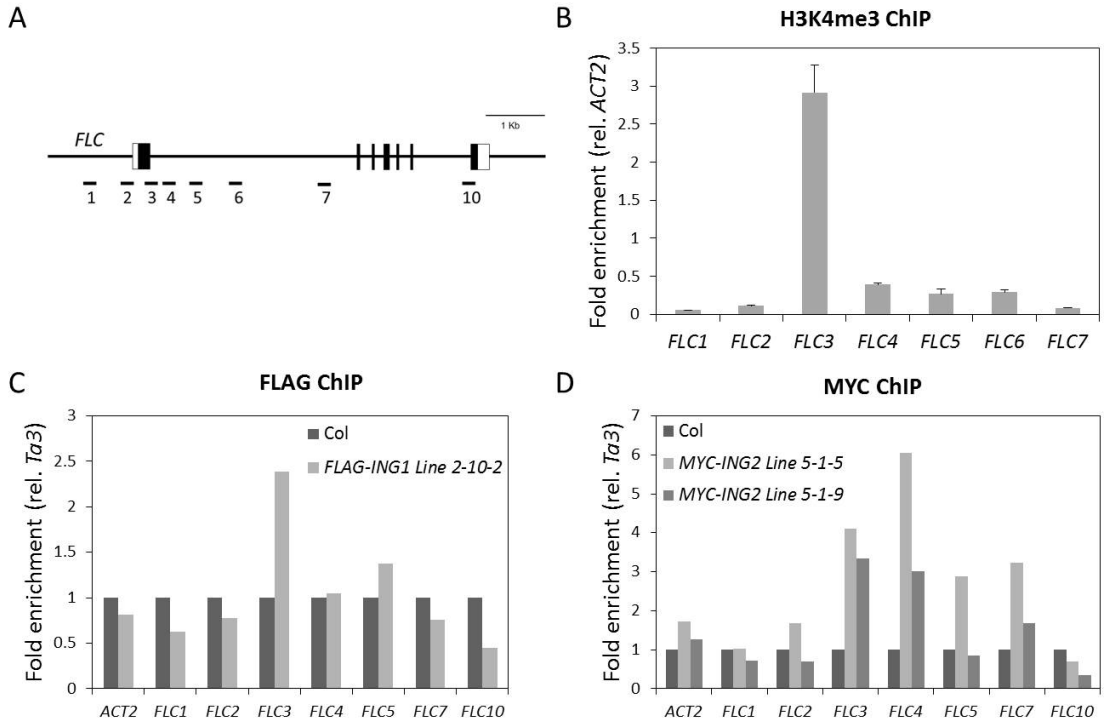

Figure 56. ING1 and ING2 bind *FLC* chromatin. (A) Schematic representation of the *FLC* gene and the regions analyzed by qPCR. **(B)** H3K4me3 distribution along *FLC* in Col plants. **(C)** ChIP analysis of FLAG-ING1 enrichment at the *FLC* locus with a 35S::FLAG-ING1 line versus Col plants. **(D)** ChIP analysis of Myc-ING2 enrichment at the *FLC* locus with two 35S::Myc-ING2 line versus Col plants. In **(B)**, **(C)** and **(D)** the amount of immunoprecipitated genomic fragments was measured by qPCR and the enrichment was calculated by normalizing the values of immunoprecipitated DNA to inputs and to an internal control (*ACT2* or *Ta3*). In **(C)** and **(D)** values in Col plants were set to 1.

3.4.2. ING2 binds FT *chromatin*

Our molecular expression data indicate that while *ing1* mutants display an upregulation of *FT*, mutations in *ING2* have the opposite effect in the expression of this gene. Besides, our genetic data indicate a quasi-epistasis of *FT* over *ING2* (Fig. 53). To test if *FT* is a

direct target of ING1 and/or ING2, we decided to assess the binding of these proteins to the chromatin of this floral integrator gene.

To address the binding of ING1 to *FT* chromatin, we performed ChIP with an anti-FLAG antibody using the *ING1* overexpressing line 2-10-2, and analyzed the binding to the *FT* regions indicated in Fig. 57A. We did not clearly detect a significant enrichment in any of the regions tested (Fig 57B), suggesting that ING1 does not bind *FT*, although we cannot rule out the binding of ING1 to other genomic regions of this gene.

Next, we used the *ING2* overexpressing lines 5-1-5 and 5-1-9 to test the binding of ING2 to *FT*, performing ChIP with an anti-Myc antibody. In this case, we found an enrichment of immunoprecipitated DNA in both transgenic lines in regions *FT3* and *FT5*. These results indicate that *FT* is a direct target of *ING2*.

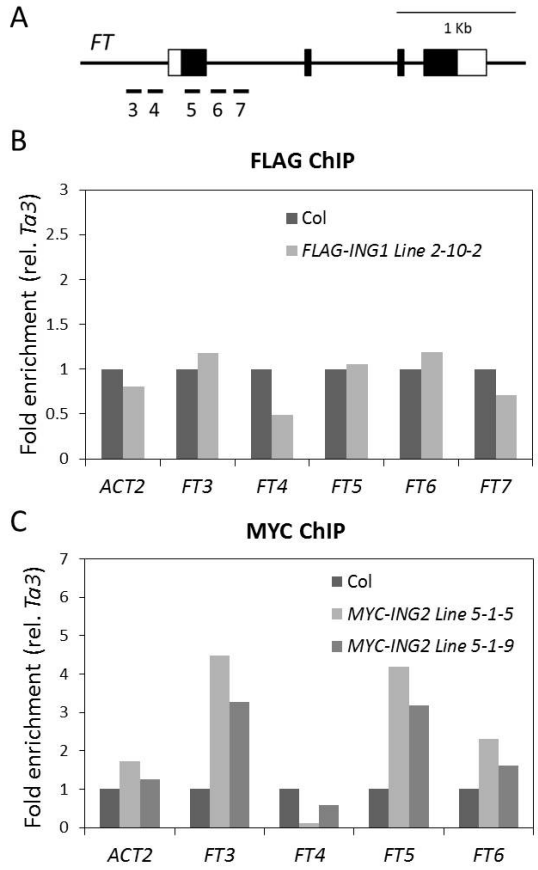

Figure 57. ING2 binds *FT* chromatin. **(A)** Schematic representation of the *FT* gene and the regions analyzed by qPCR. **(B)** ChIP analysis of FLAG-ING1 enrichment at the *FT* locus with a 35S::FLAG-ING1 line versus Col plants. **(C)** ChIP analysis of Myc-ING2 enrichment at the *FT* locus with two 35S::Myc-ING2 lines versus Col plants. In **(C)** and **(D)** the amount of immunoprecipitated DNA genomic fragments was measured by qPCR and the enrichment was calculated by normalizing the values of immunoprecipitated DNA to inputs and to an internal control (*Ta3*). Values in Col plants were set to 1.

3.4.3. Mutations in ING1 or ING2 do not affect H2A.Z deposition at FLC

Our genetic data suggest that SWR1 and the *ING1* gene regulate *FLC* expression independently while SWR1 and *ING2* gene interact genetically to regulate this repressor of flowering. To assess the possible involvement of *ING* genes in the deposition of H2A.Z, we decided to analyze the distribution of this histone variant in *FLC* chromatin using the *pHTA11::HTA11-GFP* line generated by Kumar and Wigge (2010) and described in section 2.10 . For that, this construct was introduced by genetic crosses in *ing1-1* and *ing2-2* mutant backgrounds. As in 2.10, we analyzed two genomic regions of *FLC* that are already known to

have high levels of H2A.Z (*FLC3* and *FLC10*), one region with low levels of this histone variant (*FLC7*), and also a chromatin region for a locus that does not contain H2A.Z (At4g07700) (Fig. 58A). Mutations in either *ING1* (Fig. 58B) or *ING2* (Fig. 58C) do not cause major alterations in the distribution of HTA11 across the *FLC* locus, indicating that the deposition of H2A.Z in the chromatin of *FLC* does not depend on *ING1* or *ING2*.

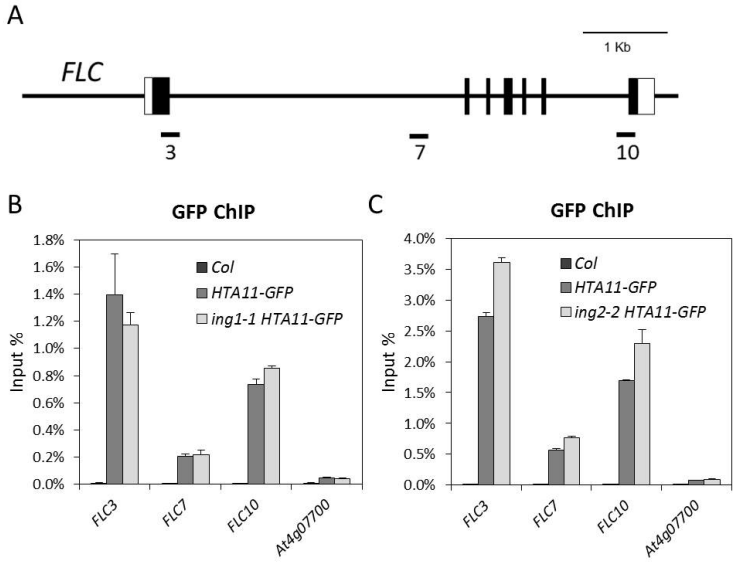

Figure 58. Mutations in *ING1* or *ING2* do not significantly affect H2A.Z deposition at *FLC* gene. (A) Representation of the *FLC* gene and the regions amplified by qPCR. Enrichment of H2A.Z at the *FLC* locus analyzed by ChIP using an anti-GFP antibody in HTA11-GFP lines in Col, *ing1-1* **(B)** and *ing2-2* **(C)** backgrounds. Col plants were used as a negative genetic control. The amount of immunoprecipitated genomic fragments was measured by qPCR and expressed as input %. A chromatin region of At4g07700 that does not contain H2A.Z was used as a negative genomic control.

3.4.4. ING1 *and* ING2 *are required to maintain high H4 acetylation levels at* FLC

Given that *FLC* is a direct target of ING1 and ING2 and that *FT* is a direct target of ING2, and that both ING proteins interact with components of the Piccolo NuA4, it is possible that they may modulate the levels of histone acetylation in the chromatin of these flowering genes. To test this hypothesis, we decided to analyze H4 acetylation levels in *ing1-1* and *ing2-2* mutants. We performed ChIP experiments using an antibody that recognizes the tetra-acetylated isoform of H4 and analyzed the regions of *FLC* and *FT* indicated in Fig. 59A, B. In the case of *FLC*, we observed a reduction in H4 acetylation levels in *ing1-1* and *ing2-2* in all four regions tested (Fig. 59C). Lower H4Ac levels are particularly conspicuous in the *FLC3* region where H4 acetylation reaches a maximum in wt plants and both ING1 and ING2 bind the *FLC* locus preferentially. In the case of *FT*, H4 acetylation levels in *ing1-1* were comparable to those observed in Col in the analyzed regions (Fig. 59D). However, we found that these levels were reduced in *ing2-2*, particularly in region *FT4*. These results indicate that *ING1* and *ING2* are required to maintain normal H4 acetylation levels in *FLC*, and that *ING2* is also required for proper H4 acetylation in the chromatin of *FT*, supporting the idea that Arabidopsis ING proteins contribute to maintain H4 acetylation levels in the chromatin of their target genes.

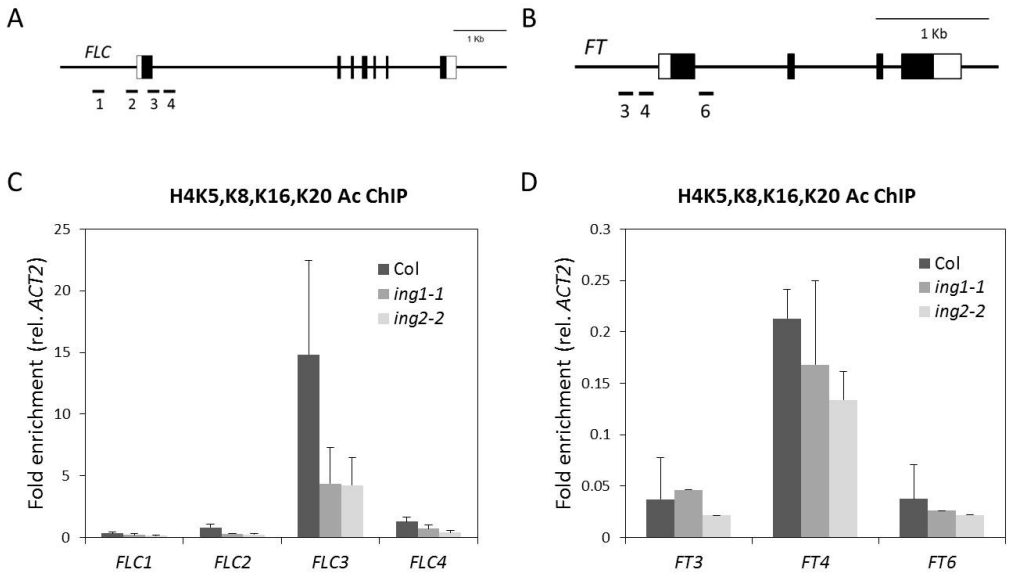

Figure 59. *ING1* and *ING2* are required to maintain high acetylation levels at flowering target genes. Schematic representation of the *FLC* **(A)** and *FT* **(B)** genes and the chromatin regions analyzed by qPCR. Distribution of H4K5,K8,K16,K20 Ac along *FLC* **(C)** and *FT* **(D)** in Col, *ing1-1* and *ing2-2* plants. The amount of immunoprecipitated genomic fragments was measured by qPCR and the enrichment was calculated by normalizing the values of immunoprecipitated DNA to inputs and to an internal control (*ACT2*).

3.5. *Transcriptomic analysis of* ing1-1*,* ing2-2 *and* ing1-1 ing2-2 *mutants*

To get a deeper insight on the processes regulated by ING1 and ING2 and their possible target genes, we performed global transcriptomic analyses by RNA-seq with Col, *ing1-1*, *ing2-2* and *ing1-1 ing2-2* plants. We isolated RNA from samples grown in LD for 10 days and harvested at ZT16. Each sample was collected in triplicate and the data from *ing1-1*, *ing2-2* and *ing1-1 ing2-2* were all compared to Col.

We found 608 genes misregulated in *ing1-1*, of which 207 were up-regulated and 401 were down-regulated. 1019 genes showed altered expression in *ing2-2* compared to Col, 572 being up-regulated and 447 being down-regulated. In *ing1-1 ing2-2* double mutant plants, the number of misregulated was the highest (1751), 1384 of them being up-regulated and 367 down-regulated. The number of misregulated genes in *ing2-2* is higher than in *ing1-1*, suggesting that ING2 has a more prominent role in the control of gene expression than ING1. This observation is in agreement with the stronger phenotypic alterations displayed by *ing2-2* mutants, compared to *ing1-1*. However, this can also be due to the fact that the *ing1-1* mutant is not a full knock out allele, and it is possible that in a plant where the expression of *ING1* is completely abolished, the number of affected genes could be much higher. We also noticed that the number of misregulated genes in the double mutant is greater than in any of the single mutants.

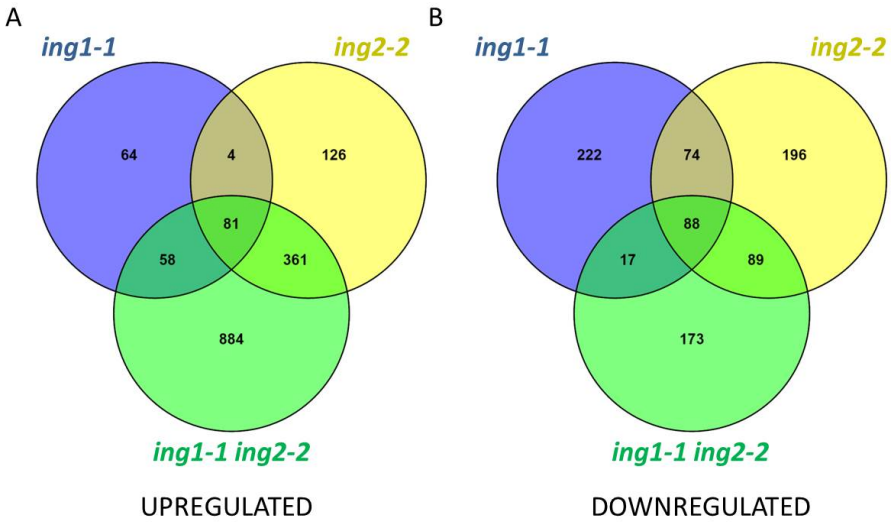

Figure 60. *ING1* and *ING2* have independent and redundant functions in the control of gene expression. Venn diagrams representing the number of upregulated **(A)** and downregulated **(B)** genes in *ing1-1*, *ing2-2* and *ing1-1 ing2-2*, compared to Col. The Venny 2.0 tool (http://bioinfogp.cnb.csic.es/tools/venny) was used to generate Venn diagrams.

To further understand the possible functional redundancy or independence between *ING1* and *ING2* in the control of gene expression, we compared the degree of overlap between the upregulated and downregulated genes in *ing1-1*, *ing2-2* and *ing1-1 ing2-2* mutants, and the results are shown using Venn diagrams (Fig. 60). We observed that 64% of the upregulated genes and 47% of the down-regulated genes in *ing1-1 ing2-2* are not misregulated in the single mutants, indicating that a high percentage of genes are regulated redundantly by ING1 and ING2. We can also observe that even though there is some degree of overlap between *ing1-1* and *ing2-2*, there are also a high number of genes that are specifically misregulated in each mutant. This analysis indicates that *ING1* and *ING2* have both redundant and independent roles in the regulation of gene expression.

We took a closer look at the most upregulated and downregulated genes in *ing1-1*, *ing2-2* and *ing1-1 ing2-2*. We represented in heatmap diagrams the 20 genes showing the strongest up and downregulation in each genotype compared to Col (Fig. 61, 62, and 63).

Figure 61. Genes differentially expressed in *ing1-1* seedlings. Shown are the 20 genes more upregulated and downregulated, respectively. From left to right: expression values expressed as log2 (*ing1-1*/wt), gene identificator (AGI number) and description of each gene.

We found that a high number of genes involved in the cold acclimation response were misregulated in these mutants, particularly in *ing1-1*. Among them, *C-REPEAT/DRE BINDING FACTOR 1* and *2* (*CBF1* and *2*), positive regulators of cold acclimation, were upregulated in *ing2-2* and *ing1-1 ing2-2*. Interestingly, a number of upstream regulators of *CBF1* and *CBF2* were downregulated specifically in *ing1-1*, including *INDUCER OF CBF EXPRESSION1* (*ICE1*), *CALMODULIN BINDING TRANSCRIPTION ACTIVATOR3* (*CAMTA3*), *PHYTOCHROME-INTERACTING7* (*PIF7*), as well as downstream effectors such as *RESPONSIVE TO DESSICATION 29A*/*COLD REGULATED78* (*RD29A*/*COR78*), *RD29B*, and other regulators of the cold acclimation response such as *KIN1* and *DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN 2* (*DREB2A*). The expression values for these genes are represented in Table 7. These results argue for a role of ING proteins in the control of cold acclimation in Arabidopsis, and the contrasting regulation of positive regulators of this process suggests that *ING1* and *ING2* functions could be opposite. Further analyses will be required to confirm a role for ING proteins in the Arabidopsis response to cold.

	ing1-1		*ing2-2*		*ing1-1 ing2-2*	
Gene	**log (*ing1-1*/Col)**	**p-value**	**log (*ing2-2*/Col)**	**p-value**	**log (*i1i2*/Col)**	**p-value**
CAMTA3	-1,078	1,79E-05	-1,116	1,13E-05	-	-
CBF1	-	-	4,179	3,78E-05	5,319	2,04E-11
CBF2	-	-	4,533	3,78E-12	4,855	1,75E-15
DREB2A	-1,032	6,46E-09	-	-	-	-
ICE1	-2,824	9,48E-06	-	-	-	-
KIN1	-1,332	1,41E-23	-	-	-	-
PIF7	-2,016	9,72E-05	-	-	-	-
RD29A	-1,035	6,05E-109	-	-	-	-
RD29B	-2,740	3,38E-30	-	-	-	-

Table 7. Genes involved in the cold acclimation response that are misregulated in *ing* mutants. Expression values expressed as log 2 (mut/Col) and p-value for each gene in *ing1-1*, *ing2-2* and *ing1-1 ing2-2* mutants.

The *ing1-1* mutant also presented an unanticipated amount of genes involved in seed germination among the upregulated genes. For instance, genes like *SEED STORAGE ALBUMIN 1*, *2*, *3*, *4* and *5*, and other members of the seed storage superfamily showed increased expression. Other germination-related genes were also upregulated, such as *ARABIDOPSIS THALIANA SEED GENE 1* (*ATS1*), *CRUCIFERIN 2* and *3* (*CRU2, CRU3*), *OLEOSIN 1-4* (*OLEO1-4*). Among the upregulated genes in *ing1-1 ing2-2*, we also found a high enrichment of genes involved in seed germination, including all the genes just mentioned and also other key regulators of this process like *ABA INSENSITIVE 3* (*ABI3*), *ABI5*, *DELAY OF GERMINATION 1* (*DOG1*), *CHOTTO 1* (*CHO1*) and *CYSTEINE PEROXIREDOXINE 1* (*PER1*). This indicates that ING proteins could also play a fundamental role in the early stages of development. Even though we found that some genes encoding seed storage proteins and *ABI5* were also upregulated in *ing2-2*, the high number of germination genes found in *ing1-1* suggests that *ING1* might have a predominant role over *ING2* in the regulation of this developmental transition. Further studies will be required to confirm this hypothesis.

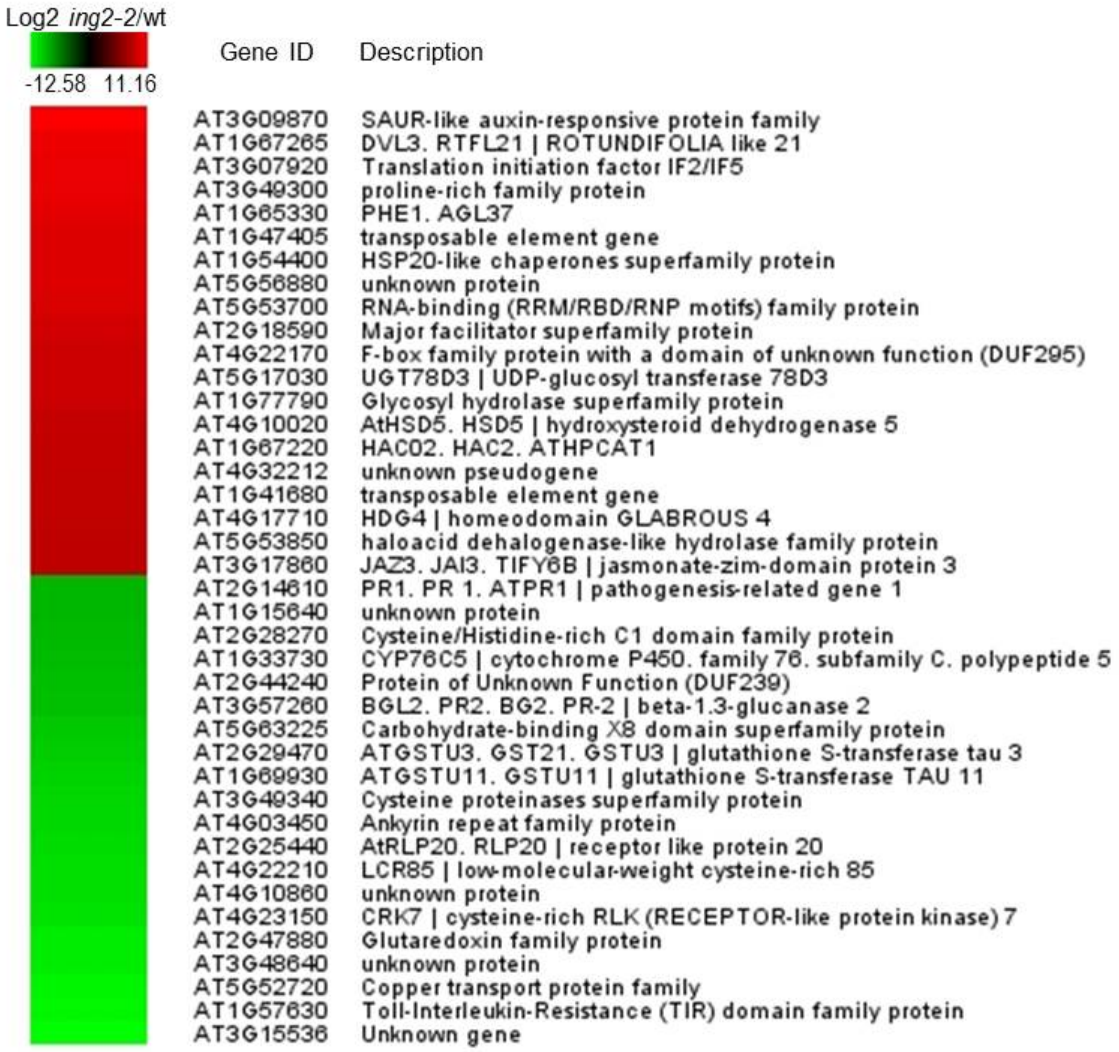

Figure 62. Genes with altered expression in *ing2-2* seedlings. Shown are the 20 genes more upregulated and downregulated, respectively. From left to right: expression values expressed as log2 (*ing2-2*/wt), gene identificator (AGI number) and description of each gene.

The analysis of misregulated genes in *ing2-2* showed a significant enrichment in transcription factors. For instance, *PHE1*, an important regulator of embryo development, *HOMEODOMAIN GLABROUS4* (*HDG4*), and the jasmonate signaling regulator *JAZ3* were among the 20 most upregulated genes (Fig. 62). Also, important regulators of the defense response like PR1 and PR2 were strongly downregulated in this mutant.

Among the downregulated genes in *ing1-1 ing2-2* we could find the cytochrome P450 member *CYP76C5*, *PLANT CADMIUM RESISTANCE 1* (*PCR1*), involved in Cd^{++} resistance (Song et al., 2004) and *CALRETICULIN 3* (*CRT3*), a chaperon-like lectin that plays a role in the quality control of two receptor-like kinases (Jin et al., 2009b; Li et al., 2009) (Fig. 63).

Figure 63. Genes with altered expression in *ing1-1 ing2-2* seedlings. Shown are the 20 genes most upregulated and downregulated, respectively. From left to right: expression values expressed as log2 (*ing1-1 ing2-2*/wt), gene identificator (AGI number) and description of each gene.

To get a global view of the processes regulated by ING1 and ING2, we analyzed the enrichment of GO functional categories among the misregulated genes in *ing1-1 ing2-2*. Regarding the biological process, we observed that the most enriched category is related to ¨regulation of transcription¨, which highlights the role exerted by ING1 and ING2 in gene transcriptional regulation, followed by the categories: “oxidation-reduction processes”, “response to chitin” and “response to abscisic acid and salicylic acid” (Fig. 64A). Regarding the molecular function, we found that most of the misregulated genes fall into the categories of transcription factors and DNA binding (Fig. 64B), which again argues for an important role of *ING1* and *ING2* as regulators of gene expression in Arabidopsis.

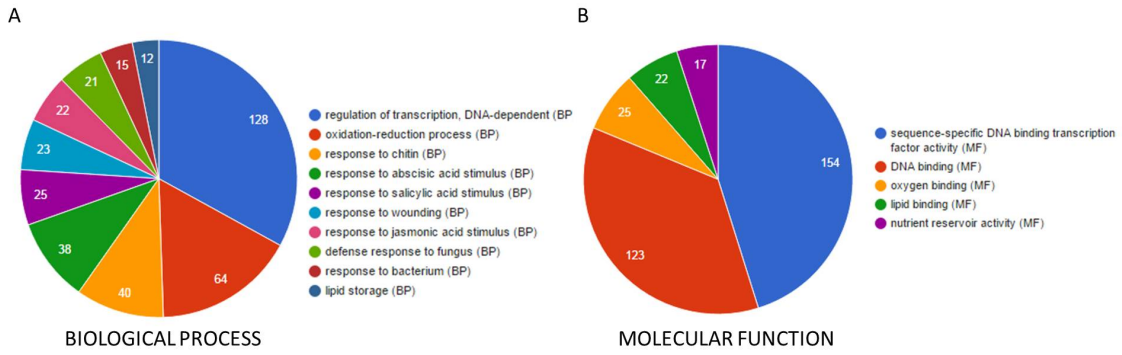

Figure 64. Enrichment of GO functional categories among misregulated genes in *ing1-1 ing2-2*. Number of genes per annotation attending to biological process **(A)** and molecular function **(B)** among misregulated genes in *ing1-1 ing2-2*.

Some genes involved in the regulation of the floral transition are also found misregulated in *ing* mutants (Table 8), according to our RNA-seq data. Most importantly, the expression of *FT* was upregulated in *ing1-1* and downregulated in *ing2-2*. This differential expression pattern is in agreement with the early flowering phenotype of *ing1-1* and the late flowering phenotype of *ing2-2* and with our previous observations regarding gene expression analysis (Sections 3.1.3 and 3.1.7). As shown in section 3.1.9, the expression of this floral integrator is also downregulated in *ing1-1 ing2-2* plants, although our RNA-seq data does not reveal significant alterations in the expression of this gene in *ing1-1 ing2-2* plants. The early flowering phenotype of *ing1-1* can also be associated to the down-regulation of *ARABIDOPSIS THALIANA GIBBERELLIN 2-OXIDASE 1* (*AtGA2ox1*). The protein encoded by this gene is capable of specifically deactivate bioactive GAs and contribute to delay the floral transition (Rieu et al., 2008). The downregulation of this gene in *ing1-1* plants could result in a higher concentration of GAs and eventually in an acceleration of flowering.

	ing1-1		***ing2-2***		***ing1-1 ing2-2***	
Gene	**log (*ing1-1*/Col)**	**p-value**	**log (*ing2-2*/Col)**	**p-value**	**log (*i1i2*/Col)**	**p-value**
AtGA2ox1	-8,593	5,61E-05	-	-	-	-
AtGA2ox2	-	-	2,633	8,51E-07	3,055	5,96E-10
FT	1,196	1,75E-26	-1,213	1,51E-12	-	-
MAF5	-	-	-	-	1,958	7,24E-36
TEM1	-	-	2,336	0	2,672	0
TEM2	-	-	2,623	1,37E-08	3,300	4,98E-16

Table 8. Genes involved in the regulation of flowering that are misregulated in *ing* mutants. Expression values expressed as log 2 (mut/Col) and p-value for each gene in *ing1-1*, *ing2-2* and *ing1-1 ing2-2* mutants.

On the other hand, we found some other flowering regulators that were misregulated in *ing2-2* and *ing1-1 ing2-2,* consistently with the late flowering phenotype of these mutants. For instance, another GA inactivating enzyme, *ARABIDOPSIS THALIANA GIBBERELLIN 2-OXIDASE 2* (*AtGA2ox2*), was upregulated in both mutants. In this case, lower GA concentrations due to increased GA inactivation in these plants could cause a delay of

flowering. We also found an increased expression of the floral repressors *TEM1*and *TEM2*in *ing2-2* and *ing1-1 ing2-2*. These proteins counteract the effect of the floral activator CO and repress the expression of *FT* in LD (Castillejo and Pelaz, 2008). It has recently been shown that TEM proteins delay the floral transition also by repressing the expression of two GA biosynthesis genes (Osnato et al., 2012). The higher expression of *TEM1* and *TEM2* in *ing2-2* and *ing1-1 ing2-2* might contribute to repress *FT* expression in these mutants and delay flowering. Lastly, we also observed that the *FLC* clade member *MAF5* was upregulated in *ing1-1 ing2-2*. *MAF5* acts as a floral repressor and its overexpression causes a delay in flowering (Ratcliffe et al., 2003). Elevated *MAF5* levels in *ing1-1 ing2-2* could also contribute to the late flowering phenotype observed in these plants.

DISCUSSION

DISCUSSION

Chromatin remodeling plays a central role in the establishment and maintenance of gene expression patterns that govern plant development. Moreover, chromatin structure provides a mechanism that ensures the stability of these expression patterns throughout mitotic cell divisions in a cell lineage (Jarillo et al., 2009). In animals, the epigenetic states that control development are established in the early stages of development. In plants, epigenetic mechanisms that control organogenesis and developmental transitions such as flowering operate post-embryonically, suggesting that chromatin remodeling may contribute to the developmental plasticity of plants.

As sessile organisms, the chances of survival of plant species depend on their ability to cope with changes in the environment. Plants can modulate their developmental programs in response to a number of endogenous and external factors, providing them with great plasticity to adapt to their surrounding environment. The time of flowering is one of the most important developmental transitions, as it is crucial to determine the reproductive success of the plant. Favorable conditions promote flowering through the activity of the floral activating pathways (Andres and Coupland, 2012). These pathways are antagonized by the floral repressors, which ensure that the floral transition does not take place until plants are in the appropriate developmental stage, and environmental conditions are optimal (Jarillo and Pineiro, 2011).

FLC encodes one of the main floral repressors in Arabidopsis, as uncovered by a large number of studies addressing the role of this gene in the control of flowering time (Crevillen and Dean, 2011). These studies have revealed that *FLC* is subjected to an extensive epigenetic regulation, and, in fact, the transcriptional control of *FLC* has become a paradigm of epigenetic regulation in plants, given the numerous chromatin remodeling events modulating the expression of this gene. Among all these activities, the SWR1 complex plays a key role in the activation of *FLC*. The SWR1-mediated exchange of histone H2A by H2A.Z is essential for *FLC* activation, as shown by the fact that mutations in SWR1 components fully suppress the late flowering phenotype conferred by an active allele of *FRI* (Choi et al., 2007; Martin-Trillo et al., 2006). The fact that the yeast SWR1-C shares several subunits with the NuA4-C, the existing functional interplay between these two complexes in yeast, and the finding that both biochemical activities have been evolutionary merged into a single complex in humans, the Tip60 complex, suggests that the relationship between these two chromatin remodeling complexes might also be conserved in other organisms.

Our analysis has revealed that there are conserved homologues for most of the NuA4 subunits in the Arabidopsis genome, suggesting the existence of a putative NuA4 complex in plants. The Epl1 subunit is a central component of yeast NuA4. Together with the catalytic subunit Esa1, Epl1 is the only essential subunit that is specific for NuA4 (Mitchell et al., 2008). Epl1 anchors the Piccolo subcomplex to the rest of the NuA4 complex and independently binds all Piccolo subunits (Chittuluru et al., 2011; Boudreault et al., 2003), acting as a platform for the assembly of this subcomplex. Apart from this structural function, yeast Epl1 has a major role in allowing the binding of NuA4 to chromatin substrates and enhancing NuA4 catalytic activity (Boudreault et al., 2003; Chittuluru et al., 2011). The interaction of Epl1 with Esa1 is of

utmost importance for NuA4 function as it stimulates Esa1 enzymatic activity towards histones (Chittuluru et al., 2011). In this work we have demonstrated the interaction of both Arabidopsis EPL1 and EPL2 with the Esa1 homolog HAM1 by Y2H assays (Fig. 54). These experiments also showed that the Arabidopsis homologs of yeast Yng2, ING1 and ING2, interact with EPL1 and EPL2, respectively (Fig. 55). In addition we found that ING1 and ING2 also interact with another putative Piccolo subunit, EAF6 (Fig. 55). These results support the existence of a Piccolo subcomplex in Arabidopsis.

Additional reports have recently demonstrated physical interactions between other NuA4 components in Arabidopsis. The catalytic subunit HAM1 has been shown to interact with the yeast Eaf3 homologues MRG1 and MRG2 (Xu et al., 2014). Also, Bieluszewski et al. (2015) performed affinity purification assays followed by tandem mass spectrometry using ARP4 and SWC4, two of the shared subunits between SWR1 and NuA4, as baits to identify *in vivo* interactors for these proteins. These authors found that a number of putative NuA4 components co-purify with ARP4 and SWC4, namely, EAF1A and B, EAF6, EPL1 and 2, HAM1, ING2, YAF9A and B, MRG1, and TRA1. The physical interaction of YAF9A and B with SWC4 and EAF1 was further confirmed by co-immunoprecipitation and BiFC assays. Altogether, these observations strongly support the conservation of a putative NuA4 complex in Arabidopsis.

Different functional modules within the yeast NuA4-C have been proposed (Auger et al., 2008; Chittuluru et al., 2011). Piccolo NuA4 can be considered as the HAT module, as it bears the catalytic activity and exists independently of the rest of the complex. It encompasses Esa1, Epl1, Eaf6 and Yng2 (Fig. 4), and is responsible for global non-targeted acetylation of the genome. The rest of subunits constitute a recruitment module that targets NuA4 to specific loci (Chittuluru et al., 2011; Auger et al., 2008) (Fig. 4). The Eaf7, Eaf5 and Eaf3 subunits form the TINTIN submodule within the recruitment modulethat also exists independently of NuA4 (Rossetto et al., 2014; Bhat et al., 2015).

To shed light on the function of this putative Arabidopsis NuA4 complex in the control of plant development and particularly flowering time, we have initiated the molecular and functional characterization of subunits belonging to this HAT complex. We have studied representative protein members of these three functional modules, focusing mainly on homologues of the Piccolo subunits, and especially in the ING1 and ING2 homologues, and their participation in the control of the floral transition.

1. The recruitment module subunit TRA1 participates in the activation of the floral transition

Yeast Tra1 is an integral component of two HAT complexes, NuA4 and SAGA (Grant et al., 1998; Saleh et al., 1998; Allard et al., 1999), and is essential for cell viability (Saleh et al., 1998). These complexes act as transcriptional co-activators, interact with general and specific transcription factors, and cooperatively activate gene expression. Tra1 seems to play two types of roles within these complexes. First, it is involved in complex assembly, as deletion mutants of Tra1 fail to associate with SAGA and NuA4 components (Knutson and Hahn, 2011), indicating that this subunit acts as a scaffold. Second, it plays a regulatory role, recruiting SAGA

and NuA4 to chromatin through the interaction with transcriptional activators (Brown et al., 2001; McMahon et al., 1998; Lang et al., 2001).

We have identified two homologues of yeast Tra1 in the Arabidopsis genome: *TRA1* and *TRA2*. *TRA1* is required to activate the floral transition in both LD and SD, as shown by the late flowering phenotype displayed by different *tra1* alleles (Fig. 6). Moreover, this late flowering phenotype is accompanied by an increase in *FLC* and a decrease in *FT* and *SOC1* expression (Fig. 7), which is consistent with a role of TRA1 in the regulation of these flowering master genes. However, *tra2* mutants did not display alterations in flowering time compared to wt plants, at least in LD (Fig. 8). It is possible that *TRA2* is not involved at all in the regulation of flowering, or that *TRA1* compensates for the absence of *TRA2*. In this case, only in a *tra1 tra2* double mutant the putative roles of *TRA2* in the regulation of flowering could be revealed. Mutations in *Tra1* and *TRRAP* are lethal in *S. cerevisiae* and mice, respectively, and therefore, a double mutant *tra1 tra2* might not be viable in Arabidopsis. However, this gene is duplicated in *S.pombe*, where two paralogous proteins are present, namely Tra1 and Tra2 (Hayashi et al., 2007). In this organism, there has been specialization of each protein, and while Tra1 associates specifically with SAGA, Tra2 associates specifically with NuA4. A tempting possibility is that, similarly to *S. pombe*, Arabidopsis TRA proteins might also have acquired specialization during evolution, and while one of them might be specific for NuA4, the other could be part of other chromatin remodeling complexes, such as Arabidopsis SAGA. Recent proteomic results by Bieluszewski et al. (2015) using ARP4 and SWC4 as baits showed that TRA1 copurified with the NuA4 subunit SWC4. Interestingly, TRA2 was not found to interact with either SWC4 or ARP4, supporting the hypothesis that Arabidopsis TRA proteins might have undergone functional divergence in evolution, although further evidence will be required to conclude that this subunit is present in other chromatin remodeling complexes.

TRA1 proteins in other organisms mediate the recruitment of NuA4 to particular loci, contributing to the specificity of the large NuA4 complex, versus the non-targeted acetylation role performed by the Piccolo NuA4 subcomplex. The interaction of TRA proteins with specific transcription factors, such as Gcn4, Hap4 and Gal4 (Brown et al., 2001) in yeast, and c-Myc, E2F and p53 (McMahon et al., 1998; Lang et al., 2001) in humans appears to be behind this ability. It would be interesting to identify potential interactors of Arabidopsis TRA1 and TRA2 to shed some light into the possible mechanisms by which Arabidopsis NuA4 is recruited to target genes.

2. Arabidopsis homologues of TINTIN subunit Eaf3 are necessary for the activation of photoperiodic flowering

Recent work in yeast has provided evidence for the existence of the TINTIN submodule within NuA4, which exists independently of the rest of the complex and is composed by Eaf7, Eaf5 and Eaf3 subunits (Rossetto et al., 2014; Bhat et al., 2015). This subcomplex carries out independent functions from NuA4 in transcription elongation, mRNA processing and mRNA quality control (Rossetto et al., 2014; Bhat et al., 2015). TINTIN is localized within the coding region of target genes and interacts with RNA Pol II, promoting the disruption of nucleosomes in its path, and ensuring the proper refolding of the chromatin after RNA pol II read-through.

The TINTIN subunit Eaf3 contains a CHD domain, which is usually present in proteins that recognize histone modifications in the chromatin. These histone "readers" participate in the recruitment of chromatin remodeling complexes that modify chromatin structure and enroll downstream effectors that eventually translate the "histone code" into patterns of gene expression (Suganuma and Workman, 2011; Mouriz et al., 2015). Transcriptionally active genes are enriched in H3K4me3 and H3K36me3. The latter accumulates at high levels in gene bodies and is linked with transcriptional elongation (Lee and Shilatifard, 2007; Shilatifard, 2006). Several proteins have been shown to recognize methylated H3K36 in different organisms (Zhang et al., 2015). However, during the initial stages of this PhD Thesis, no chromatin readers of methylated H3K36 had been identified in Arabidopsis.

Eaf3 CHD is able to specifically bind H3K36me3 (Sun et al., 2008; Xu et al., 2008a). This recognition facilitates RPD3-mediated deacetylation in gene bodies, preventing cryptic initiation of transcription (Carrozza et al., 2005; Joshi and Struhl, 2005; Keogh et al., 2006; Li et al., 2007). Eaf3 is involved in the regulation of the balance of H3 and H4 acetylation levels. Loss of *Eaf3* function does not lead to changes in global levels of H3 and H4 acetylation, but it rather affects their distribution. Yeast strains with mutations in *Eaf3* show increased histone acetylation in coding sequences of genes and decreased acetylation in promoters, so global levels of acetylation remain unchanged (Reid et al., 2004). Increased acetylation in coding regions usually correlates with aberrant initiation of transcription in gene bodies (Carrozza et al., 2005). The combined action of Eaf3 and the PHD-containing protein Rco1 recruits the RPD3S HDAC complex to coding regions, which subsequently deacetylates histones (Li et al., 2007). This process relies on the recognition of H3K36me3 by the CHD of Eaf3. However, it has also been proposed that in the context of NuA4 Eaf3 regulates H4 acetylation in promoter regions (Reid et al., 2004).

Given the key role of this protein in the chromatin-mediated regulation of gene expression, we examined the role of the putative Arabidopsis homologues of Eaf3, MRG1 and MRG2, which are very similar in sequence to yeast Eaf3 and its homologues in other organisms like human and Drosophila MRG15 (Fig. 17). Structural studies have shown that four amino acids in yeast Eaf3 CHD, Tyr23, Tyr82, Trp84 and Trp88, are directly involved in the formation of an aromatic cage that mediates the recognition of H3K36me3 by this protein (Sun et al., 2008; Xu et al., 2008a). These four residues are conserved in the CHD of Drosophila and human MRG15 as well as in Arabidopsis MRG1 and MRG2 (Fig. 17). Also, *in silico* predictions of MRG1 and MRG2 structure show that both proteins are very similar to each other and to Eaf3 (Fig. 18).

Sequence and structural similarity suggest functional redundancy between *MRG1* and *MRG2*. In fact, *mrg1* and *mrg2* single mutants show very subtle alterations in flowering time. However, the *mrg1 mrg2* double mutant displayed a very late flowering phenotype, only under LD (Fig. 20). This suggests that similarly to other NuA4 subunits, *MRG1* and *MRG2* seem to play redundant roles as activators of the floral transition. Also, the fact that this phenotype was only observed under LD and not in SD, suggests that the regulation of flowering mediated by the MRGs is related to the photoperiod pathway. The delay in flowering time observed in *mrg1 mrg2* double mutants was accompanied by a strong downregulation of *FT*, the main output of the photoperiod pathway, and also of *SOC1* which acts downstream of *FT*, further supporting

this hypothesis (Fig. 21). In addition, this delay in flowering time seems to be almost completely dependent on *FT*, since the simultaneous loss of function of *mrg1* and *mrg2* barely affects flowering time in an *ft-10* mutant background (Fig. 22). These results point to a role of the MRGs in the photoperiodic regulation of flowering, mainly upstream of *FT*.

In yeast, Eaf3 function is linked to H3K36 methylation mediated by the histone methyltransferase Set2. Strains carrying mutations in *Eaf3* and *Set2* and strains with an alanine substitution at H3K36 (H3K36A) display similar phenotypes in terms of histone acetylation in coding regions of genes (Joshi and Struhl, 2005; Carrozza et al., 2005). This observation can be explained by the fact that H3K36me3 is the histone mark that recruits Eaf3 to the chromatin, modifying the acetylation status. To understand if Arabidopsis MRG1 and MRG2 functions also depend on H3K36me3 we introgressed the *mrg1* and *mrg2* mutations in a background deficient for *SDG8*. The analysis of the *mrg1 mrg2 sdg8* triple mutant showed that the deficiency in *SDG8* completely suppresses the late flowering phenotype of *mrg1 mrg2*, and indicated that SDG8-mediated H3K36 methylation is required for the function of these proteins (Fig. 23). This supports a working model where Arabidopsis MRGs also act through the recognition of this epigenetic mark to recruit downstream effectors.

Our findings were further corroborated by two articles published simultaneously during the course of this PhD thesis, in which the role of Arabidopsis MRG1 and MRG2 in flowering was characterized (Bu et al., 2014; Xu et al., 2014). These studies provided experimental evidence that MRG1 and MRG2 bind H3K36me2/3, and also H3K3me3, therefore acting as readers of these marks. Consistently, the authors also confirmed that the function of the MRG proteins depends on SDG8-mediated methylation of H3K36. Similarly to our observations, these studies also revealed a late flowering phenotype of *mrg1 mrg2* that correlated with decreased expression of *FT* under LD. Both works proved the direct binding of MRG2 to *FT* regulatory regions by ChIP approaches, and in addition, Bu et al. (2014) showed that this binding depends on an active SDG8. These authors also presented evidence that MRG2 physically interacts with CO, and both proteins enhance each other's binding to *FT* chromatin, providing a plausible explanation for the photoperiodic regulation of flowering by MRG proteins. On the other hand, Xu et al. (2014) showed that the interaction of MRG1 and 2 with HAM1 and HAM2 is required to maintain normal levels of H4K5 acetylation at the *FT* locus. Altogether, these data support a model where, under inductive photoperiods, CO recruits MRG1/2 to *FT*. Both MRG proteins bind the chromatin of this floral integrator through the CHD-mediated recognition of SDG8-methylated H3K36, leading to H4K5 acetylation by HAM1, possibly in the context of NuA4.

This model explains easily the late flowering phenotype of *mrg1 mrg2* mutants under LD. However, we also observed a strong downregulation of the floral repressor *FLC* in this double mutant (Fig. 21), which was confirmed by Xu et al. (2014). This is not the first time that a chromatin remodeling protein is reported to regulate concurrently these two genes with contrasting functions in the control of flowering time. For instance, CLF and SWN mediate the deposition of H3K27me3 at *FT* and *FLC* loci and repress their expression (Jiang et al., 2008; Oh et al., 2008). The possible regulation of *FLC* by MRG1/2 remained unexplored, and given the relevance of the epigenetic regulation of this floral repressor, we investigated it in more detail. By performing ChIP experiments we demonstrated that MRG2 binds the chromatin of *FLC*,

indicating that this gene is a direct target regulated by MRG2 (Fig. 25). Given the role of the MRGs in the modulation of H4 acetylation levels at *FT*, we evaluated whether histone acetylation was also affected at *FLC* in *mrg1 mrg2* plants. Surprisingly, we found no reduction in either H3 or H4 acetylation levels at this locus (Fig. 26). Instead, H4 acetylation was slightly increased in a region just downstream of the TSS. As histone acetylation frequently correlates with transcriptional activation, additional regulatory elements besides increased H4Ac are likely to play a part in the reduced levels of *FLC* expression observed in *mrg1 mrg2* plants. As discussed previously, MRG homologues in other organisms are involved in the RPD3-mediated histone deacetylation in coding regions, which in turn prevents cryptic transcriptional initiation within gene bodies. A tempting possibility is that the increased H4 acetylation levels observed in *mrg1 mrg2* double mutants at the *FLC* locus could lead to the production of cryptic transcripts in *FLC*, and this outcome would be independent of NuA4 activity. An alternative explanation could be related to the fact that two lncRNAs repress *FLC* expression during vernalization, *COLDAIR* and *COOLAIR* (Berry and Dean, 2015). *COLDAIR* is a sense lncRNA that is transcribed from *FLC* intron 1 (Heo and Sung, 2011). A defect in histone deacetylation in *FLC* regions surrounding the promoter of *COLDAIR* might result in increased transcription of this lncRNA and lead to the *FLC* down-regulation observed in *mrg1 mrg2* mutants. Further studies will be required to test this hypothesis.

This model implies that MRG1 and MRG2 would function primarily in coordination with HDACs to mediate *FLC* deacetylation. As discussed above, yeast Eaf3 is present in both NuA4 and RPD3 complexes. There are several histone deacetylases in Arabidopsis belonging to the RPD3 type of HDACs (Hollender and Liu, 2008). Among them, HDA6, HDA9 and HDA5 have been shown to regulate flowering time (Yu et al., 2011; Kim and Sung, 2013; Kang et al., 2015; Luo et al., 2015). HDA6 and HDA5 function together with FLD and FVE to form HDAC complexes that repress *FLC* expression, and mutations in these genes confer a late flowering phenotype (Yu et al., 2011; Gu et al., 2011; Luo et al., 2015). HDA6 has been shown to physically interact with MRG2 (Xu et al., 2014). We therefore hypothesized that HDA6 could be involved in the MRG1/2-mediated deacetylation of *FLC*. However, the triple mutant *mrg1 mrg2 axe1-5* flowered later that the parental mutant plants, revealing that HDA6 and the MRGs regulate flowering through different genetic pathways. However, the role of the MRGs in the regulation of *FT*, downstream of *FLC*, and the likely role of HDA6 in the regulation of additional flowering genes might mask a possible genetic interaction of the *MRG1* and *MRG2* genes with *HDA6* in the regulation of *FLC*. Furthermore, at present we cannot rule out that other RPD3 HDACs are involved in this process again masking putative genetic interactions between *MRG* genes and *HDA6*.

The distribution of other histone marks associated with transcription, such as H3K4me3 and H3K27me3 did not show significant alterations in *mrg1 mrg2* plants. In yeast, members of the trimer Eaf5/7/3 show a strong genetic interaction with SWR1 (Rossetto et al., 2014; Krogan et al., 2004). Therefore, we explored the possible relationship between the MRGs and the SWR1-C. The analysis of *HTA11*-encoded H2A.Z distribution at *FLC* in *mrg1 mrg2* plants did not show significant differences compared to the control, indicating that MRG proteins do not affect H2A.Z incorporation by SWR1. However, we cannot exclude the possibility that the H2A.Z acetylation might be affected in the *mrg1 mrg2* double mutant and could contribute to explain the downregulation of *FLC* observed in these plants.

Compelling evidence indicates that the TINTIN functional module might be evolutionary conserved. Human cells do not present homologues of Eaf5, but the Eaf7 homolog MRGBP and the Eaf3 homolog MRG15 have been shown to interact and coexist as a dimer independently of Tip60 (Cai et al., 2003; Kirkwood et al., 2013). We have not found Eaf5 homologues in Arabidopsis, but At1g26470 may encode a putative homolog of Eaf7. Besides, MRG2 interacts with HAM1, HAM2 and EPL2 (Xu et al, 2014; our group, unpublished results), while MRG1 co-purifies with SWC4 (Bieluszewski et al., 2015). These interactions support the notion that the MRGs might be part of Arabidopsis NuA4. However, it would be interesting to analyze if these proteins, together with EAF7, also exist in an independent submodule similar to TINTIN, and if so, how is their association with NuA4 regulated.

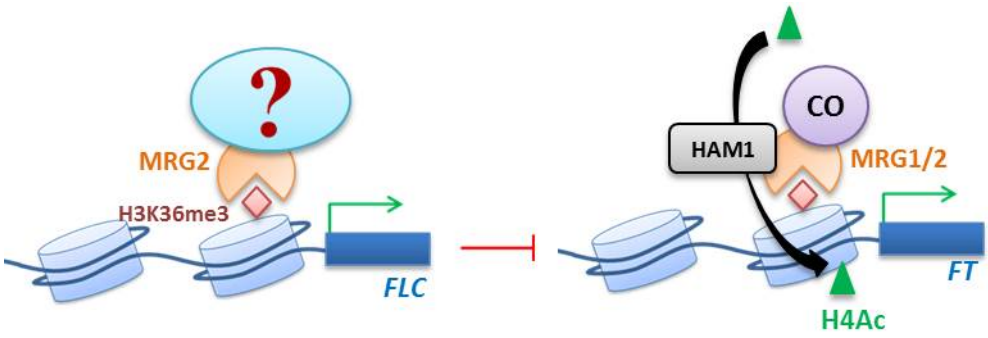

Figure 65. Working model for MRG1/2 roles in the regulation of the floral transition. MRG1 and 2 cooperate with CO and HAM1 to regulate the chromatin-mediated photoperiodic activation of *FT* expression by modulating the levels of H4Ac in this locus. MRG2 also binds the chromatin of *FLC* and activates its expression through a yet unknown mechanism.

We and others have shown that *MRG1* and *MRG2* play redundant roles in the control of the floral transition and that they participate in the transcriptional regulation of *FLC* and *FT* (Fig. 65). As discussed above, the CHD domain in MRG proteins is able to recognize and bind H3K36me2/3, an epigenetic mark present in the body of transcriptionally active genes and associated with the elongating form of Pol II (Shilatifard, 2006; Carrozza et al., 2003). The large number of Arabidopsis genes that carry this mark suggests that MRG1 and 2 could be involved in the regulation of many processes. To address this question we performed transcriptomic analyses with *mrg1 mrg2* plants by RNA-seq. Our analysis showed that 1084 genes were misregulated in these plants. This number appears somehow small, considering that MRG1 and MRG2 are so far the only proteins known to be able to read H3K36 methylation in Arabidopsis and that the number of genes carrying the H3K36me3 mark is close to 16000 in this plant species (Roudier et al., 2011). However, it is also noteworthy that the number of misregulated genes found in *sdg8* mutants varies a lot depending on the developmental stage analyzed: Xu et al. (2008b) reported only 142 misregulated genes in 6-day-old *sdg8-2* seedlings, while the number of misregulated genes determined in 2-week-old leaves from *sdg8-2* was around 1500 (Tang et al., 2012). This suggests that this histone modification is probably rather dynamic, and therefore, the relatively low number of misregulated genes in *mrg1 mrg2* plants can be due to the particular developmental stage at which the samples were taken.

The number of misregulated genes found in our RNA-seq data for *mrg1 mrg2* plants involved in flowering time regulation was quite low. Consistent with our qPCR analysis we found downregulated the expression of *FT*. In addition to that, we could only detect upregulated the expression of the floral repressors *TEM1* and *TEM2*. These results confirm that the late flowering phenotype observed in *mrg1 mrg2* plants seems to be specifically due to the misexpression of *FT*. However, the GO analysis of the RNA-seq data revealed that a high number of misregulated genes were involved in the regulation of transcription, most of them being sequence-specific transcription factors involved in DNA binding, highlighting the important regulatory role of MRG proteins.

The defense response against pathogens could also be altered in *mrg1 mrg2* plants. Many of the differentially expressed genes in the RNA-seq dataset fell into categories related to defense against pathogens such as fungi, or bacteria, and also other categories indirectly related to defense, such as response to jasmonic and salicylic acid. In fact, the two most down-regulated genes were plant defensins, and among the down-regulated genes we can also find major defense regulators like *PR1* and *2*. Interestingly, *sdg8* mutant plants also display alterations in the defense against pathogens (Berr et al., 2012; De-La-Pena et al., 2012) and an altered hormone response (Wang et al., 2014a). This supports a role for SDG8-mediated H3K36 methylation and subsequent recognition by MRG1/2 in the regulation of defense genes in Arabidopsis. In this regard, it will be interesting to test the response of *mrg1 mrg2* plants to pathogen attacks.

We have revealed a role for *MRG1* and *MRG2* in the promotion of flowering that is dependent on SDG8-mediated H3K36me3 deposition and activation of *FT*. These two chromatin remodeling proteins play an important regulatory role, controlling the expression of genes involved in many biological processes, such as defense against pathogens and hormone regulation. The function of these proteins, and particularly their role as flowering activators, seems to be evolutionary conserved in other plant species. The rice homolog MRG702 also acts as a reader of H3K4me3 and H3K36me3 and its loss of function leads to late flowering and affects brassinosteroid biosynthesis and signaling (Jin et al., 2015). Thus, a deeper understanding of the functions performed by MRG proteins in Arabidopsis could help to improve the yield of crops species.

3. Piccolo NuA4 subunits are also involved in the regulation of the floral transition

Piccolo NuA4 is responsible for global non-targeted H2A and H4 acetylation (Boudreault et al., 2003). The catalytic activity of Piccolo resides in Esa1, and its activity as a free complex accounts for the maintenance of global histone acetylation levels throughout the genome. This is essential for cell viability, and accordingly, deletion of *Esa1* results in lethality. Apart from Esa1, Piccolo NuA4 encompasses Epl1, Eaf6 and Yng2.

As discussed above, Epl1 is a central component of yeast NuA4 given its structural role as a scaffold for the assembly of the other Piccolo subunits, its ability to anchor Piccolo to the rest of NuA4, and its function of stimulating the catalytic activity of Esa1. We have found that

there are two *Epl1* homologues in Arabidopsis, *EPL1* and *EPL2*. Interestingly, loss of function mutations for both genes cause late flowering in LD and at least *epl1* mutants also flower late in SD (Fig. 9), indicating that these proteins in Arabidopsis are involved in the activation of the floral transition. Furthermore, both proteins may control the expression of the flowering master regulators *FLC* and *FT*, as evidenced by the misregulation of these genes in *epl1* and *epl2* mutants under LD (Fig. 11). We have attempted to generate an *epl1 epl2* double mutant to uncover possible functional redundancy between these two genes. However, after analyzing a large number of plants in the F2 population from the genetic cross *epl1 x epl2*, no such double mutant plant could be obtained. Considering the crucial role of yeast Epl1 in the maintenance of Piccolo NuA4 structure, it is possible that the concurrent loss of function of Arabidopsis *EPL1* and *EPL2* might cause a severe disruption (or abolishment) of NuA4 assembly and/or activity that could result in lethality.

Eaf6 is also a component of yeast Piccolo NuA4. Unlike Tra1, Eaf3 and Epl1 homologues, the Arabidopsis homologue of Eaf6 is a single-copy gene. The analysis of *eaf6* mutants revealed that they display pleiotropic phenotypic alterations, including small rosette size, leaf curling, and reduced chlorophyll concentration. These alterations resemble those displayed by other NuA4 mutants, such as *yaf9a yaf9b* double mutants (Choi et al., 2011; Bieluszewski et al., 2015) and *SWC4* knockdown lines (Gómez-Zambrano et al, unpublished), supporting a role for the putative Arabidopsis NuA4 complex in the regulation of different developmental processes.

We have found that Arabidopsis EAF6 functions in the promotion of flowering. This role was only revealed under SD photoperiods, as *eaf6-2* mutants did not show alterations in flowering time or in the expression of *FLC* and *FT* under LD conditions (Fig. 13, 14). It is possible that the photoperiodic activation of flowering operating in LD could mask any possible defects in floral activation in *eaf6-2* mutants under long photoperiods. In SD, however, they displayed a very late flowering phenotype and, in some experiments a complete inability to flower under these conditions (Fig. 13). Consistent with this observation, the expression of *FLC* was increased at day 20 in *eaf6*-2 compared to wt, while the expression of *FT* decreased at day 20 and day 25 in this mutant (Fig. 14).

The possible role of EAF6 in the regulation of *FLC* led us to introduce the *eaf6-2* mutation in a *FRI* background, an allelic variant that causes high levels of *FLC* expression (Crevillen and Dean, 2011). We found that *eaf6-2 FRI* plants displayed a much later flowering phenotype than *FRI*, and most of the plants analyzed failed to flower. However, *FLC* levels in the *eaf6-2 FRI* plants were comparable to the *FRI* parental line, which indicates that the enhancement of the late flowering phenotype of these plants is independent of *FLC* (Fig. 16). Altered expression of other flowering genes, such as *FT* or *SOC1*, could cause the late flowering phenotype of these *eaf6 FRI* mutant plants, although this possibility remains to be tested.

Exogenous GA could partially rescue the late flowering phenotype of *eaf6-2* in SD (Fig. 15). This observation indicates that these mutants are able to respond to GA and therefore the GA perception signaling pathway is not affected. The delay in flowering could be caused either by defects in GA biosynthesis or by misregulation of genes acting upstream of the known GA targets in SD, *SOC1* and *LFY*. However, the fact that GA-treated *eaf6-2* plants still flower later

than GA-treated Col plants suggests that EAF6 also has a promoting role in flowering that is independent of GA.

As mentioned above, yeast Epl1 enhances the enzymatic activity of Esa1 but not its affinity for chromatin. The subunit responsible for the preference of Piccolo NuA4 for chromatin over free histones is Yng2. In fact, it has been proposed that Yng2 is necessary for the proper positioning of Esa1 in an orientation that favors catalysis on histone tails (Chitturuluru et al., 2011), which highlights the relevance of this subunit for the function of NuA4. The role of Yng2 as a chromatin-interacting protein is evidenced by the presence of a PHD domain. The PHD motif represents a big family of epigenomic effector domains, and it mediates the specific binding of proteins to posttranslational histone modifications. This interaction promotes the recruitment of chromatin remodeling factors and components of the transcriptional machinery to underlying loci modulating gene expression (Li and Li, 2012). The PHD domain in human BROMODOMAIN AND PHD FINGER TRANSCRIPTION (BPTF) and ING2 were the first to be functionally characterized, and were shown to specifically bind H3K4me3 (Pena et al., 2006; Li et al., 2006; Wysocka et al., 2006). Since then, the number of characterized PHD proteins that recognize H3K4me2/3 has increased. Recent reports have shown that PHD domains also have the ability to recognize other histone marks, such as H3K9me3, H3K36me3, H3K14Ac or unmodified H3K4 and H3R2. PHD fingers can recognize more than one histone mark, and tandem PHDs can bind several marks, underscoring the versatility of this family of proteins. The binding of PHD proteins to specific epigenetic marks in the chromatin mediates the recruitment of effectors that can activate or repress the expression of underlying genes, such as HATs or HDACs.

PHD proteins are conserved in eukaryotic organisms, including plants. The Arabidopsis proteome contains at least 83 proteins with canonical PHD fingers (Lee et al., 2009), and a number of them have been shown to regulate several developmental processes (Mouriz et al., 2015). As mentioned in the Introduction section, the PHD-containing VEL family of proteins have a crucial role in the silencing of *FLC* after exposure to low temperatures. Other PHD proteins have been shown to regulate several aspects of plant development. For instance, MALE MEIOCYTE DEATH1, MALE STERILITY1 and the ASH1-RELATED 3 SET-domain protein are essential for the completion of meiosis and post-meiotic processes (Yang et al., 2003; Borg and Twell, 2010; Thorstensen et al., 2008). OBERON1 and 2 are involved in the specification of the vasculature and primary root meristem (Thomas et al., 2009; Saiga et al., 2012), while PICKLE prevents the expression of embryonic traits in seedlings (Ogas et al., 1999). ORC1, a subunit of the origin recognition complex is also a transcriptional regulator (de la Paz Sanchez and Gutierrez, 2009). Also, the plant-specific PHD-containing proteins EBS and SHL control flowering time through the chromatin-mediated regulation of *FT* and *SOC1*, respectively (Gomez-Mena et al., 2001; Pineiro et al., 2003; Lopez-Gonzalez et al., 2014). Members of the Alfin1-like (AL) family of proteins are involved in the germination process. AL proteins interact with PRC1 components and mediate the switch from H3K4me3- to H3K27me3-enriched chromatin to silence seed developmental genes and promote germination (Molitor et al., 2014). AL6 has also been to modulate root development in low Pi conditions (Chandrika et al., 2013).

The importance of yeast Yng2 for NuA4 function and the prominent role of PHD proteins in Arabidopsis development prompted us to characterize the putative Yng2 homologs in Arabidopsis, focusing on flowering time. We identified two homologs of Yng2 in Arabidopsis, ING1 and ING2, which share sequence similarity with ING proteins from other species, like yeast Yng1 and Yng2, and members of the human ING family (Fig. 31). The similarity was higher in the region of the PHD, highlighting the importance of this domain for ING function. A hydrophobic cage in the structure of the PHD finger, formed by aromatic (Trp and Tyr) and hydrophobic (Met) residues mediates the recognition and binding of INGs to H3K4me3 (Champagne and Kutateladze, 2009). These residues, conserved in human and yeast ING proteins, were also present in Arabidopsis ING1 and ING2 (Fig. 31), consistently with reports showing the specific binding of ING1 and ING2 to H3K4me3 (Lee et al., 2009). These observations suggest that the PHD-mediated recognition of this epigenetic mark is key for the function of these proteins in Arabidopsis.

3.1. ING1 *is required for the* FLC*-mediated repression of flowering*

Mutations in *ING1* accelerated flowering in LD, but caused no flowering alterations in SD, indicating that this protein acts as a repressor of flowering under long photoperiods. The fact that *ing1-1* plants do not show alterations in flowering under SD might indicate a function of ING1 in the photoperiod pathway. However, we cannot rule out the possibility that this is a consequence of *ing1-1* not being a null allele, and in a plant that completely lacks ING1, a role for this protein in SD might be revealed.

At the molecular level, the early flowering phenotype of the *ing1-1* mutant is accompanied by changes in the expression of master regulators of flowering. These plants show decreased expression of *FLC*, *MAF1*/*FLM*, *MAF2*, *MAF4* and *MAF5*, and increased expression of *FT* and *SOC1* (Fig. 35), which indicates that *ING1* participates, directly or indirectly, in the regulation of these genes. In agreement with these observations, the analysis of the *ing1-1 flc-3* double mutant revealed an epistatic relationship of *FLC* over *ING1*, since double mutant plants did not show any further acceleration of flowering or *FT* induction compared to the single mutants (Fig. 45), indicating that the effect of *ING1* depends on *FLC*. Also, when introduced in a *FRI* background, the *ing1-1* mutation accelerated flowering and caused a decrease in the expression of *FLC* (Fig. 46). Altogether these observations corroboratethat *ING1* positively regulates *FLC* expression. We also tested the genetic relationship between *ING1* and *FT* by generating an *ing1-1 ft-10* double mutant (Fig. 53). The *ing1-1* mutation did not accelerate flowering in an *ft-10* background, indicating that *ING1* represses flowering upstream of *FT*. Since *FT* acts downstream of *FLC* in the regulation of flowering initiation, this result is in agreement with our previously revealed genetic interaction of *ING1* with *FLC*.

Given the functional relationship between SWR1 and NuA4 in yeast and the role of SWR1 in *FLC* regulation, we tested a possible genetic relationship between SWR1 components and *ING1*. Double mutant combinations of *ing1-1* with *esd1-10* and *swc6-1* showed an additive flowering phenotype and downregulation of *FLC*, demonstrating that the role of *ING1* is

independent of SWR1 activity (Fig. 47). Accordingly, our ChIP results showed that the *ing1-1* mutation does not affect H2A.Z deposition at *FLC* (Fig. 58), supporting the functional independence between *ING1* and SWR1.

Since ING proteins recognize H3K4me3, we analyzed if the phenotype of *ing1-1* depends on the activity of proteins that modulate the levels of this mark. In particular, ATX1 and ATXR7 are histone methyltransferases that deposit H3K4me3 in the chromatin of *FLC* and mediate its transcriptional activation (Pien et al., 2008; Tamada et al., 2009; Berr et al., 2009). The analysis of mutant combinations with *ing1-1* showed that *ATX1* and *ATXR7* are epistatic to *ING1* (Fig. 50). This is consistent with the idea that the effect of ING1 on the regulation of *FLC* depends on H3K4me3 deposition in the chromatin of this gene, and in genetic backgrounds with impaired H3K4 methylation at *FLC*, the absence of *ING1* does not cause further alterations in flowering time.

It is not uncommon to find tandem PHD fingers or several PHD-containing proteins acting in cooperation within the same chromatin remodeling complex. For instance, the two PHD-containing proteins JADE and ING4 are found in the human HBO HAT complex (Doyon et al., 2006). While the JADE PHD finger is required for the recruitment of HBO to the chromatin, the binding of ING4 PHD finger to H3K4me3 increases the acetyltransferase activity of HBO at target promoters (Hung et al., 2009; Saksouk et al., 2009). For this reason, we investigated the relationship of *ING1* and *ING2* with other "readers" of the methylation status of H3K4 known to regulate flowering time. EBS is a PHD-containing protein that is able to bind H3K4me3, and it is involved the chromatin-mediated repression of *FT* by binding the chromatin of this gene and modulating the levels of histone acetylation (Lopez-Gonzalez et al., 2014). Like *ing1-1*, the *ebs* mutant displays an early flowering phenotype and elevated levels of *FT* expression. The analysis of *ing1-1 ebs* double mutant plants revealed that *EBS* is epistatic to *ING1*, as *ing1-1 ebs* flowered at the same time as *ebs*, both in LD and SD (Fig. 51). This indicates that *ING1* and *EBS* regulate flowering in the same genetic pathway and that *EBS* acts downstream of *ING1*, which is consistent with our previous data showing that *FT* was required for the early flowering phenotype of *ing1-1*.

Our genetic data clearly indicate that the *ING1* genetically interacts with *FLC* and *FT*. In addition, our molecular data showed that ING1 participates in the regulation of the expression of both genes. Therefore, we investigated if this regulation was direct by testing the binding of the ING1 protein to the chromatin of these master regulators of flowering. Our ChIP experiments showed that ING1 binds the chromatin of *FLC*, and as expected, it binds an H3K4me3-enriched region (Fig. 56), consistent with the notion that this protein functions as a "reader" of this mark. However, no binding of ING1 to *FT* chromatin was detected (Fig. 57). Given that ING1 interacts with NuA4 subunits, we tested if there were changes in H4 acetylation levels in the chromatin of *FLC* and *FT* in *ing1-1* plants. Our ChIP results showed that mutations in *ING1* lead to a clear decrease in H4 acetylation at *FLC*, but cause no changes in the levels of this mark at *FT* chromatin (Fig. 59).

Altogether, these results are compatible with a model in which ING1 represses flowering by directly regulating *FLC* expression (Fig. 66). The H3K4me3-dependent binding of ING1 to *FLC* chromatin drives H4 acetylation in this locus, possibly in the context of NuA4,

allowing its full transcriptional activation under LD, contributing to maintain low levels of *FT* expression until the conditions are optimal for flowering. This role of ING1 as a flowering repressor is in line with the proposed roles of other putative NuA4 subunits, like HAM1 and 2 or the shared subunit with SWR1-C YAF9A (Xiao et al., 2013; Zacharaki et al., 2012), which have also been shown to repress flowering through the activation of *FLC*.

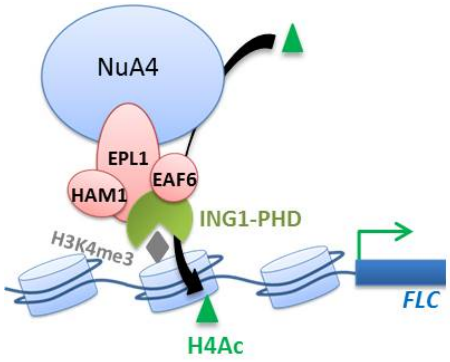

Figure 66. Working model for the chromatin-mediated repression of *FLC* by ING1. ING1 interacts with the putative NuA4 components EPL1 and EAF6 and binds the chromatin of *FLC* in H3K4me3-enriched regions to activate its expression by maintaining H4 acetylation levels in this locus.

3.2. *ING2 promotes flowering through the activation of* FT

We have isolated two mutant alleles of *ING2*, the weak allele *ing2-1* and the strong allele *ing2-2* (Fig. 36). Interestingly, the phenotypic analysis of these mutants revealed an opposite phenotype to *ing1-1* mutants under LD, as mutations in *ING2* cause a delay in flowering time (Fig. 37). Under SD, the same trend was observed in *ing2* mutants, with *ing2-1* plants flowering later and *ing2-2* plants failing to flower and dying after the production of a high number of rosette leaves. These observations indicate that ING1 and ING2 have opposite functions in the regulation of the floral transition: while ING1 functions as a repressor of flowering under LD, ING2 acts promoting flowering initiation in LD and SD. Interestingly, ING proteins in other organisms have also been shown to have opposite functions. Specifically, yeast Pho23 and Yng1 play opposite roles to Yng2 in the p-53 dependent activation of transcription (Nourani et al., 2003).

The expression of the floral integrators *FT* and *SOC1* was reduced in *ing2-2* plants, in agreement with their late flowering phenotype. However, no significant changes in *FLC* and only slight increases in *MAF1*/*FLM*, *MAF2* and *MAF4* expression levels were detected in these mutants (Fig. 40). Although *ing2-2* plants did not show significant alterations in the expression of *FLC*, the *flc-3* mutation suppressed the late flowering phenotype of *ing2-2* (Fig. 41), which indicates that this phenotype depends on an active *FLC*. Also, mutations in *ING2* delay flowering and cause an upregulation of *FLC* in *FRI* backgrounds (Fig. 46). It is possible that the regulation of *FLC* by ING2 may require full activation of *FLC*, and therefore, it was only revealed in a *FRI* background and not in Col. To test this hypothesis, it would be informative to introgress the *ing2-2* mutation in other genetic backgrounds with high *FLC* levels, such as the autonomous pathway *fve* or *fca* mutants.

Our genetic analysis also revealed interactions between *ING2* and genes encoding SWR1 components. Mutations in *ESD1*/*ARP6* and *SWC6* fully suppressed the late flowering phenotype of *ing2-2*, even showing further acceleration of flowering and further

downregulation of *FLC* in the case of *ing2-2 swc6-1* (Fig. 48, 49). This indicates that the phenotype of *ing2-2* depends on *FLC* activation by SWR1, and is consistent with our previous results showing that mutations in *FLC* suppress the phenotype of *ing2-2*. However, mutations in *ING2* do not affect the H2A.Z deposition activity of SWR1 in *FLC* (Fig. 58). We also analyzed the genetic relationship between *ING2* and *FT* (Fig. 53). The *ing2-2 ft-10* double mutant flowered only slightly later than *ft-10*, indicating that the function of ING2 as an activator of flowering depends mainly on *FT*.

Our genetic analysis of *ING2* with *ATX1* and *ATXR7* rendered different results to those obtained with *ING1*. Double mutant combinations of *ing2-2* with *atx1-2* and *atxr7-2* showed an intermediate phenotype (Fig. 50), suggesting that *ING2* and *ATX1*/*ATXR7* function in independent regulatory pathways controlling flowering time. This distinct interaction with ATX genes is also consistent with the independent functions of both *ING* genes in the control of flowering time. In addition, mutations in *EBS* suppressed the late flowering phenotype of *ing2-2*, under LD and SD (Fig. 52), indicating that *EBS* and *ING2* regulate flowering through the same genetic pathway. It is possible that the upregulation of *FT* caused by the *ebs* mutation could overcome the downregulation of this gene observed in *ing2-2*, leading to the early flowering phenotype observed in *ing2-2 ebs* plants.

The observed genetic relationship between *ING2* and *FT*, and the dependence on a fully active *FLC* for the late flowering phenotype of *ing2-2* prompted us to test the binding of the ING2 protein to the chromatin of these genes. Our ChIP experiments showed that ING2 binds the chromatin of *FLC*, particularly in regions that are enriched in H3K4me3 (Fig. 56). We also found that, consistent with our genetic and expression data, ING2 binds *FT* (Fig. 57), demonstrating that *FLC* and *FT* are direct targets of ING2.

Given the interactions of ING2 with other Piccolo subunits, and the results showing that ING1 is required for maintaining H4 acetylation levels at *FLC*, it is possible that ING2 could also regulate acetylation levels in the chromatin of its target genes. We tested this hypothesis by ChIP approaches and found reduced levels of H4 acetylation in *FLC* and *FT* in *ing2-2* plants (Fig. 59). These results, together with the findings rendered by Y2H experiments, suggest that the regulatory mechanism of ING2 might involve the NuA4-mediated H4 acetylation in their target genes. However, the changes in H4 acetylation observed in *FLC* in *ing2-2* plants do not correlate with changes in the expression of this gene. The activation of *FLC* by other regulatory factors might compensate the decrease in H4 acetylation caused by the *ing2-2* mutation and give rise to the unaltered *FLC* expression observed in *ing2-2* plants. In this regard, it will be interesting to analyze possible changes in histone acetylation in *FLC* in *ing2-2 FRI* plants, which show upregulated expression of this floral repressor.

Our results have uncovered a role for ING2 as an activator of the floral transition, which is consistent with the function of most of the other NuA4 subunits analyzed in this work, and show that ING2 directly binds regulatory regions of *FLC* and *FT* (Fig. 67), modulating the expression of the latter. Even though mutations in *ING2* do not cause an alteration in *FLC* expression, our genetic analyses with *FLC* and SWR1 mutants indicate that the late flowering phenotype of *ing2-2* mutants depends on activation of *FLC*. Although future experiments will be required to shed light on how ING2 regulates *FLC*, the late flowering phenotype of *ing2-2*

can be explained by its direct regulation of *FT*. The effect of ING2 on flowering depends mainly on *FT* and, in fact, in backgrounds with high levels of *FT* such as *ebs*, or in conditions that promote the upregulation of *FT*, such as warm growing temperatures, the phenotype of *ing2-2* is fully suppressed.

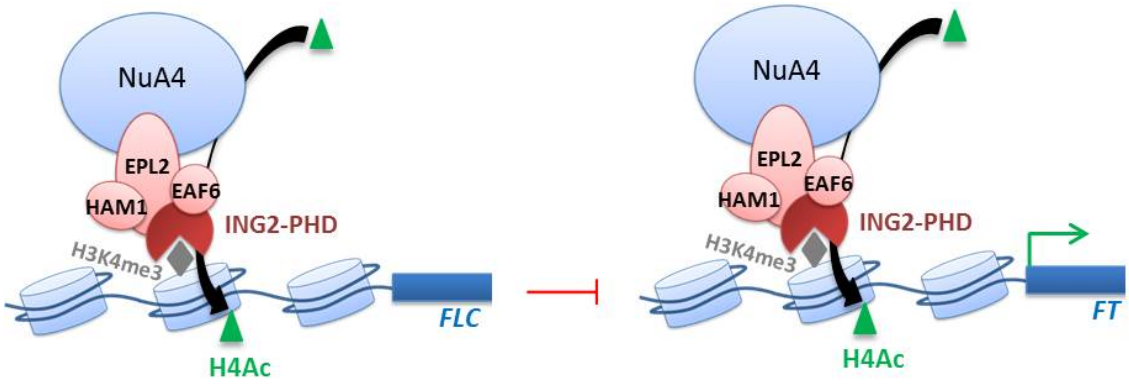

Figure 67. Working model for the roles of ING2 in the regulation of *FLC* and *FT*. ING2 interacts with EPL2 and EAF6 and binds the chromatin of *FLC* and *FT*. ING2 could be required to maintain normal H4 acetylation levels in these loci and is involved in the transcriptional activation of *FT*.

3.3. ING1 and ING2 play independent and redundant roles in development

Apart from its late flowering phenotype, the *ing2-2* mutant also showed defects in flower development, such as abnormal number of petals and disrupted floral architecture (Fig. 38), as well as altered expression of floral meristem determination and floral organ specification genes like *FUL* and *PI*, indicating that *ING2* might also have a role in the regulation of flower formation. Moreover, the phenotypic characterization of the *ing1-1 ing2-2* double mutant revealed that these proteins have redundant functions in development. These plants showed reduced silique size and lower chlorophyll concentration compared to the single mutants. They also displayed defects in gametophyte development that resulted in the appearance of aborted seed in the siliques (Fig. 43). However, *ING1* and *ING2* must also play non-redundant roles, as some of the phenotypic alterations shown by the double mutant were only present in *ing2-2* plants, such as reduced rosette size and flower defects. This indicates that *ING2* has independent functions from *ING1* in the regulation of these developmental traits. Regarding flowering, the functions of *ING1* and *ING2* are not redundant, since the *ing1-1 ing2-2* double mutant showed a late flowering phenotype comparable to that of *ing2-2* (Fig. 43). This epistatic relationship indicates that *ING2* acts downstream of *ING1* in the regulation of the floral transition, consistent with the role of ING2 in the regulation of *FT* expression previously discussed. The expression of *FLC* and *FT* genes in *ing1-1 ing2-2* follows the same pattern as in *ing2-2* (Fig. 42), confirming the predominant role of *ING2* over *ING1* in the regulation of flowering time.

Our RNA-seq data confirmed that ING1 and ING2 play independent and redundant roles also at a global gene expression level. These experiments revealed a number of genes specifically misregulated in each mutant, and also many genes that are redundantly regulated by ING1 and ING2. The number of differentially expressed genes in *ing1* and *ing2* plants suggests that *ING2* seems to have a more prominent role in the regulation of gene expression. Transcriptional analyses with stronger *ing1* alleles might allow confirming this conclusion. However, in some processes like the cold acclimation response or germination, *ING1* seems to have a predominant role over *ING2*. Our transcriptomic analysis also indicates that the INGs play a key role in the regulation of gene expression, as DNA-binding transcription factors are the most frequent category among the genes differentially expressed in the *ing1 ing2* double mutant. This is consistent with results obtained in other species, showing the involvement of ING proteins in the regulation of multiple processes like the promotion of apoptosis, DNA damage repair, cell proliferation, hormone responses, or regulation of tumor growth (Shi and Gozani, 2005; Aguissa-Toure et al., 2011).

Overall, the results obtained in this work support a role for NuA4 in the promotion of the floral transition in Arabidopsis, evidenced by the late flowering phenotype conferred by mutations in most of its putative subunits. This is in contrast with the phenotypes of loss-of-function mutants of other NuA4 subunits, such as HAM1/2 or YAF9A. These subunits have been shown to be involved in the repression of flowering through the transcriptional activation of *FLC* (Xiao et al., 2013; Zacharaki et al., 2012). In this study, we have provided evidence that MRG2, ING1 and ING2 regulate *FLC* directly, and TRA1, EPL1 and EPL2 are also involved direct or indirectly in the regulation of *FLC*. Interestingly, the yeast counterparts of many of the subunits characterized in this work, including TRA1, EAF6, MRG and ING proteins, are not exclusive to NuA4, but shared with other chromatin remodeling complexes. Thus, mutations in any of these subunits could affect the function of several chromatin remodeling activities and result in different transcriptional outcomes in genes involved in the regulation of flowering. Alternatively, different subunits might confer NuA4 specificity for different loci and explain the contrasting flowering phenotypes observed. It is also possible that different NuA4 subunits may have predominant roles in different cell types or in diverse developmental stages. Further experiments will contribute to shed light on the specific functions of NuA4 components in the regulation of the floral transition.

CONCLUSIONS

CONCLUSIONS

1. The Arabidopsis genome encodes two homologues of Tra1, AtTRA1 and AtTRA2. While TRA1 participates in the activation of flowering in LD and SD and regulates the expression of *FLC FT* and *SOC1*, loss-of-function mutations in *TRA2* do not cause alterations in flowering time under LD.
2. Two homologues of *Epl1* exist in Arabidopsis, namely *EPL1* and *EPL2*. Both proteins mediate the promotion of flowering and regulate the expression of *FLC* and *FT*.
3. *AtEAF6* encodes the only Arabidopsis homolog of yeast Eaf6, which participates in the activation of flowering under SD. The late flowering phenotype displayed by *eaf6* mutants is accompanied by an increased expression of *FLC*, concomitantly with diminished *FT* levels of expression.
4. The homologues of *Eaf3* in Arabidopsis, *MRG1* and *MRG2*, encode CHD-containing proteins that play redundant roles in the photoperiodic activation of flowering. These proteins are required for the activation of *FLC* and *FT*, and their mechanism of action is dependent on SDG8-mediated methylation of H3K36.
5. *FLC* is a direct target of MRG2, and loss-of-function mutations in *MRG1* and *MRG2* cause alterations in the acetylation levels in the chromatin of this gene, but not in the incorporation of the histone variant H2A.Z.
6. MRG1 and MRG2 participate in the transcriptional regulation of genes related to multiple biological processes.
7. The Arabidopsis homologues of Yng2, ING1 and ING2, are PHD-containing proteins that play opposite roles in the regulation of flowering time differentially regulating the expression of the flowering genes such as *FLC*, *FT* and *SOC1*.
8. ING1 and ING2 proteins bind the chromatin of *FLC* and are required to maintain proper H4 acetylation levels in this locus. ING2 also binds *FT* and regulates H4 acetylation in the chromatin of this locus.
9. ING1 and ING2 physically interact with core components of the Piccolo NuA4 subcomplex.
10. ING1 and ING2 have both independent and redundant functions in Arabidopsis development, and participate in the control of genes involved in a variety of biological processes.

CONCLUSIONES

1. El genoma de *A*rabidopsis codifica dos proteínas homólogas a Tra1, AtTRA1 y AtTRA2. TRA1 participa en la activación de la floración en día largo y día corto y regula la expresión de *FT* y *SOC1*, mientras que mutaciones en *TRA2* no provocan alteraciones en el tiempo de floración en día largo.
2. Existen dos homólogos de *Epl1* en Arabidopsis, *EPL1* y *EPL2*, que codifican proteínas muy similares. EPL1 y EPL2 tienen funciones en la activación de la floración y regulan la expresión de *FLC* y *FT*.
3. *AtEAF6* codifica el homólogo en Arabidopsis de Eaf6, que promueve la activación de la floración en día corto. El fenotipo de floración tardía de mutantes *eaf6* va acompañado de un incremento en la expresión de *FLC*, concomitante con una disminución en la expresión de *FT*, y se rescata parcialmente por GA.
4. Los homólogos de *Eaf3* en Arabidopsis, *MRG1* y *MRG2*, codifican proteínas con un dominio CHD que tienen funciones redundantes en la activación fotoperiódica de la floración. Estas proteínas son necesarias para la activación de *FLC* y *FT* y su mecanismo de acción involucra la metilación de H3K36 mediada por SDG8.
5. MRG2 se une directamente a *FLC* y mutaciones en *MRG1* y *MRG2* provocan alteraciones en los niveles de acetilación en la cromatina de este gen, pero no en la incorporación de H2A.Z.
6. MRG1 y MRG2 participan en la regulación de la expresión de genes relacionados con diversos procesos biológicos, como por ejemplo la defensa frente a patógenos.
7. Los homólogos de Yng2 en Arabidopsis, ING1 e ING2, son proteínas con un dominio PHD que tienen funciones opuestas en la regulación del tiempo de floración.
8. Las proteínas ING1 e ING2 se unen a la cromatina de *FLC* y son necesarias para mantener unos niveles de acetilación de H4 adecuados en este locus. ING2 también se une a *FT y* regula la acetilación de H4 en dicho locus.
9. ING1 e ING2 interaccionan físicamente con componentes centrales del subcomplejo Piccolo NuA4.
10. *ING1* e *ING2* tienen tanto funciones independientes como redundantes en el desarrollo de Arabidopsis y participan en la regulación de diversos procesos biológicos.

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APPENDIX

Publications directly related to the PhD Thesis

Mouriz A., López-González L., Jarillo J.A., and Piñeiro M. (2015) **PHDs govern plant development.** *Plant Signaling & Behavior*. 10(7):e993253

Komar DN, **Mouriz A**., Jarillo J.A., and Piñeiro M. (2015) **Chromatin immunoprecipitation assay for the identification of plant protein-DNA interactions *in vivo*.** *Journal of Visualized Experiments*. In press.

Other publications during this PhD Thesis

Castrillo G, Sánchez-Bermejo E, de Lorenzo L, Crevillén P, Fraile-Escanciano A, Tc M, **Mouriz A**, Catarecha P, Sobrino-Plata J,Olsson S, Leo Del Puerto Y, Mateos I, Rojo E, Hernández LE, Jarillo JA, Piñeiro M, Paz-Ares J, Leyva A. (2013) **WRKY6 Transcription Factor Restricts Arsenate Uptake and Transposon Activation in Arabidopsis.** *The Plant Cell*. **25**:2944-2957.

López-González L., **Mouriz A**., Narro-Diego L., Bustos R., Martínez-Zapater J.M., Jarillo J.A., and Piñeiro M. (2014) **Chromatin-Dependent Repression of the Arabidopsis Floral Integrator Genes Involves Plant-Specific PHD-Containing Proteins**. *The Plant Cell*. **26**:3922-3938

Lázaro A., **Mouriz A.**, Piñeiro M., and Jarillo J.A. (2015) **Red Light-Mediated Degradation of CONSTANS by the E3 Ubiquitin Ligase HOS1 Regulates Photoperiodic Flowering in Arabidopsis.** *The Plant Cell*. **27**(9): 2437-54